Esmolol
Cardioplegic effect beyond -blockade

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Awarding institution:
King's College London

Esmolol: cardioplegic effect beyond β-blockade

The British Heart Foundation Clinical PhD Studentship

of

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DEDICATION

I would like to dedicate this work to

My wife who always has faith in me.

My father whose influence made me the man I am.

My mother who taught me the true meaning of sacrifice.

Adib and Bana, my life time companions.

Alia and Nadia, the joy of my life.

ACKNOWLEDMENTS

I would like to thank Dr. Linda McLatchie for her help and support in the patch clamping studies, Dr. Sonya Bardswell in her assistance in the skinned myocytes studies and Dr. Anthony Chambers to his contribution to the Langendorff perfusion experiments.

I would, also, like to thank Professor Mike Shattock for his valuable advice and long corridor discussions, which always added to my knowledge in heart physiology.

I am grateful for Professor Jonathan C Kentish's contribution, advice and support throughout my working period on this project.

And finally, I would like to express my respect and gratitude to Dr. David John Chambers for his efforts, skills and style. He has always been a great supervisor, teacher and friend.

ABSTRACT

The use of hyperkalaemic cardioplegia in cardiac surgery induces depolarised arrest and it is currently the gold standard for myocardial protection. Although hyperkalaemia is by far the most commonly used clinically, it has been established that depolarised arrest has detrimental effects due to sodium and calcium loading. This triggered interest in establishing alternative means of cardioplegia.

Esmolol, an ultra-short acting β-blocker, was found to induce cardioplegic arrest at high (millimolar) concentrations and offers improved protection to hyperkalaemic arrest. Unlike other alternative cardioplegic agents, esmolol has been used, safely, clinically in high doses, which make it clinically relevant. However the arrest by esmolol in Langendorff perfused hearts isolated from any catecholamine background cannot be explained by its β-blocking mode of action. The aim is to establish the arresting mode of action of esmolol. Pilot data suggested that esmolol has a myofilament desensitising as well as an inhibitory effect on the calcium transient (Ca_{tr}) in the isolated ventricular myocyte.

Esmolol reduced the Ca_{tr} at high concentration (IC_{50}=~250μm). This was independent from the β-blocking effect. The effect on the sarcoplasmic reticulum (SR) was excluded by blocking the SR effect using thapsigargin and the negative inotropic effect of esmolol was maintained. Using skinned rat myocytes, esmolol did not induce myofibril desensitisation. The effect of esmolol on the L-type calcium channels was studied using patch clamp techniques. Esmolol was found to inhibit the L-type calcium channels (IC_{50}=~450μm). Esmolol also inhibited the fast Na channels (IC_{50}=~150 μm). The arrest by esmolol was caused by direct inhibition of the myocardium and not by inhibiting the conductive system only using paced Langendorff perfused hearts. It was concluded that esmolol induces arrest by blocking sodium and Calcium channels. It was last established that, in the Langendorff perfused rat heart, adding a K^+ channel opener, adenosine 0.25mM, to esmolol 0.6 mM offers better protection to St. Thomas' Hospital cardioplegia (STH) with 1 hour recovery LVDP of 69±3.7 and 51±4.6 (% of control, $p<0.01$) in 4-hour ischaemia with room temperature multiple infusion cardioplegia.

PUBLICATIONS, PRESENTAIONS, AWARDS AND INTELLECUAL PROPERTY

Publications:

Published papers

- Fallouh HB, McLatchie LM, Shattock MJ, Chambers DJ, Kentish JC. Myocardial arrest and protection with esmolol: Characterization of the diastolic arrest mechanism, ***Cardiovasc Res*** *2010 Aug 1;87(3):552-60. Epub 2010 Feb 22.*
- Chambers DJ, Fallouh HB; Cardioplegia and Cardiac Surgery: Pharmacological arrest and cardioprotection during global ischemia and reperfusion; ***Pharmacol Ther****. Jul;127(1):41-52. Epub 2010 Apr 14*
- Fallouh HB, Chambers DJ; The safety of using millimolar doses of lidocaine as cardioplegia, ***Interact CardioVasc Thorac Surg*** *2007;6:176.*
- Fallouh HB, Chambers DJ; Is blood versus crystalloid cardioplegia relevant? Significantly improved protection may require new cardioplegic concepts! ***Interact CardioVasc Thorac Surg*** *2008;7:1162-1163*
- Chambers DJ, Fallouh HB; New approaches to cardioplegia: Alternatives to hyperkalemia *(book chapter publication titled: "New solutions for the heart; an update in advanced perioperative protection"* ***Springer Verlag book****, Dec 2010*

Published Abstract

- Fallouh HB, McLatchie LM, Shattock MJ, Chambers DJ, Kentish JC. Esmolol As A Cardioplegic Agent; An Effect Beyond ß-blockade; ***Circulation.*** *2007;116:II_323-II_324.*
- Fallouh HB, McLatchie LM, Kentish JC, Shattock MJ, Chambers DJ; Sodium and calcium channel blockade by esmolol; ***J Cardiothorac Vasc Anesth.*** *2008**; Jun; 22;3:S36.***
- Fallouh HB, Bardswell SC, Chambers DJ, Kentish JC; Esmolol has a negative inotropic action independent from its beta-blocker effect; ***J Mol Cell Cardio****l; 2007; 42; S24-S36.*
- Fallouh HB, McLatchie LM, Bardswell SC, Shattock MJ, Chambers DJ, Kentish JC; Myocardial arrest by esmolol: negative inotropy induced by calcium and sodium channel blockade**; *J Mol Cell Cardiol*** *2008; 44: S49-S50.*
- Fallouh HB, Kentish JC, Chambers DJ; Does esmolol only act as a ß-blocker? A novel mode of action at higher doses in rats, ***Eur J of Anaesth****,2007;24:S41;42*

Prizes & Grants Awarded:

- **The Ronald Edwards Medal for best scientific paper;** Annual Meeting of the Society for Cardiothoracic Surgery in Great Britain and Ireland, Bournemouth, 24/03/2009. Polarised arrest, how far from clinical application.
- **Kings' College London Technology Development Fund**; 19/03/09 £40K+£50K awarded for the development of a polarising cardioplegic solution using a combination of esmolol and adenosine.

Intellectual Property

Patents number: WO 2,010,097,595

Title: Compositions for use in cardioplegia comprising esmolol and adenosine

Inventor(s): Fallouh Hazem B, Kentish, Jonathan C, Chambers David J.[GB]

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LIST OF ABREVIATIONS

A/V	Atrio-ventricular
BDM	2,3-Butanedione monoxime
BNF	British National Formulary.
Ca_{tr}	Ca^{2+} transient
DMSO	Dimethyl sulfoxide
EAC	Esmolol + Adenosine Cardioplegia
GHK	Goldman-Hodgkin-Katz
$I_{Ca,L}$	L-type Ca^{2+} channel current
I_{Na}	Fast Na^{+} channel current
KHB	Krebs Henseleit solution
LVDP	Left ventricular developed pressure
LAC	Lidocaine + Adenosine Cardioplegia
ms	Milliseconds
NMR	Nuclear magnetic resonance
RyR	Ryanodine receptors
SERCA	Sarco/endoplasmic reticulum Ca^{2+} ATPase
SL	Sarcomere length
SR	Sarcoplasmic reticulum
SS	Sarcomere shortening
STH2	St. Thomas' Hospital Cardioplegia No.2
SVC	Superior vena cava
TTX	Tetrodotoxin

CHAPTER 1. INTRODUCTION

1.1 Ischaemia in cardiac surgery

The heart is different from other organs due to the fact that it is continuously beating and has very high flow of blood circulating through it (the total blood volume of the body passes through the heart in about half a minute). During cardiac surgery, only limited procedures can be done on the heart while it is working e.g. off pump coronary surgery, mitral valvotomy, etc. To be able to do anything more than that we need to stop the heart contracting and empty the heart cavities. This is achieved by clamping the aorta and separating the heart from the systemic circulation, which offers a bloodless quiescent surgical field. Clamping the aorta, however, results in cessation of blood supply to the heart muscle through the coronary arteries, exposing the whole heart to ischaemia. This is known as 'global ischaemia' and has to be differentiated from regional ischaemia in the context of ischaemic heart disease and myocardial infarction. Global ischaemia, if prolonged will cause irreversible damage to the heart muscles. Different therapeutic measures known as myocardial protection strategies have been used to decrease the detrimental effect of global ischaemia on the heart and extend the myocardial tolerance to global ischaemia before irreversible damage is sustained.

1.2 History of cardiac surgery and the development of myocardial protection

The first successful surgical intervention on the heart was reported by Dr. Ludwig Rehn, who sutured a puncture wound in the right ventricle of a stab victim in September 1896 (Rehn 1897). Thereafter, operating on a beating heart became standard practice for heart wounds and valve stenosis. However, due to the technical difficulty of operating on a beating heart in a very bloody surgical field and in order to perform more complicated surgery, it was essential to arrest the heart and stop the blood flow through it. This

meant having to deal with the implication of abolishing the cardiac output for the length of the procedure, which would cause irreversible brain injury if it lasted for more than 2-3 minutes in normothermia (Jarcho 1975). The period of clamping the aorta was extended to 10 minutes by using whole body hypothermia, which was long enough to perform the first open heart surgery (Lewis and Taufic 1953) without permanent brain damage. The development of cardiopulmonary bypass in 1953 allowed surgeons to arrest the heart for longer times without major detrimental effects on the brain (Pastuszko and Edie 2004), this is by fully anti-coagulating the blood and draining the venous return away from the heart which provides an empty heart in order to operate on it. Subsequently, the blood is pumped into an oxygenator and filtered. The blood is then returned to the arterial circulation after having been oxygenated and would have bypassed the heart and the lungs. This is why it is called the cardiopulmonary bypass. By cross-clamping the aorta proximal to the blood return from the bypass machine. The heart becomes completely bloodless while maintaining blood supply to the rest of the body including the all important brain supply. This offered surgeons longer time to be able to operate the heart without having to worry about perfusing the brain.

Subsequently, it was recognised that inducing arrest and cessation of blood flow to the myocardium for longer periods than a few minutes in order to perform complicated surgery would expose the heart to irreversible ischaemic damage. Therefore, surgeons realised that now the brain is perfused with the cardiopulmonary bypass, it is the heart, which needs protection against global ischaemia. Hypothermia was the first and the most commonly used method of myocardial protection against ischaemia during cardiopulmonary bypass. At this stage the only method to arrest the heart was ischaemia-induced arrest where contraction stops due to energy depletion. This method was suboptimal because it resulted in faster depletion in energy stores in the cardiac myocytes and therefore faster initiation of irreversible injury as demonstrated by Hearse and colleagues much later (Hearse et al. 1974). Although this was not the driving hypothesis for Melrose's work, in 1955, he introduced the concept of chemical reversible cardiac arrest using a high potassium citrate solution in a canine model in order to avoid the problem of air embolisation when working on a beating or fibrillating heart (Melrose et al. 1955). This was the first concept of chemical myocardial

protection; however, the term "cardioplegia" was first used in 1957 (Lam et al. 1957). It became clear soon after that potassium citrate solution proposed by Melrose was shown to have detrimental effects on the myocardium leading to myocardial necrosis and increased risk of mortality and, consequently, the use of potassium-based cardioplegia became unpopular in the late 1950s (McFarland JA 1960). Alternative methods of myocardial protection were described, such as intermittent cross-clamp fibrillation and coronary occlusion (McFarland JA 1960). However, the use of cardioplegia was maintained in Germany with the development of the Bretschneider solution, which was Na-poor and Ca-free (Bretschneider et al. 1975). It was used clinically by Sondergaard and colleagues (Sondergaard et al. 1975) with success, becoming one of the first solutions to be used routinely in clinical practice. Bretschneider published his findings almost exclusively in the German literature and, hence, the English-speaking scientific communities were generally unaware of this development until much later publications in English. In the early 1970s, studies showed that the detrimental effects of the Melrose solution were a result of the increased osmolality from the high citrate concentration rather than the high potassium levels (Tyers et al. 1975). These results along with others revived the concept of cardioplegia in general and hyperkalaemia in particular. Studies in the USA by Gay and Ebert (Gay and Ebert 1973) and in the UK by Hearse and Braimbridge at St. Thomas' Hospital (Hearse et al. 1976) lead to the development of a cardioplegic solutions based on moderately elevated potassium concentrations that was shown to be effective and safe. These solutions were considered as "extra-cellular type solutions", due to their high sodium concentration compared with the Bretschneider solution, which has low sodium and classified as an "intra-cellular type solution". Hearse and colleagues continued to optimise their moderately high potassium cardioplegia by optimising the potassium and other ionic concentrations such as the use of magnesium at optimal concentrations (Hearse et al. 1978) and adding procaine as a membrane stabiliser (Hearse et al. 1981). The results of this systematic research, which was aimed at optimising the concept of arrest using high potassium was the development of the St. Thomas' Hospital cardioplegia solution No.1 (Table 1.1 Comparison between the composition of Bretschneider and St. Thomas' Hospital solutions (Modified from Chambers (Chambers and Hearse 2001)). It was first used

clinically in 1975 (Braimbridge et al. 1977). The success of this solution in protecting the myocardium against global ischaemia and reperfusion injury, identified as the "stone heart phenomenon" (Katz and Tada 1972), made it very widely used. Subsequently, the original formula of the St. Thomas' Hospital No.1 solution was slightly modified (Hearse et al. 1981) creating the St.Thomas' No.2 solution, (Table 1.1); it was registered with the Federal Drug Authorisation (FDA) in the USA and, rapidly, became by far the most widely used cardioplegic solution worldwide and it remains the gold standard crystalloid cardioplegic solution. sThe introduction of cardioplegia including the St. Thomas' Hospital solution has definitely changed the practice in cardiac surgery very rapidly (Figure 1.1). Up to 1977, ischaemic arrest along with cross-clamp fibrillation and hypothermia were the methods of choice for 94% of surgeons in the USA with only 6% using potassium cardioplegia. By 1982, 99% of these surgeons were using cardioplegia (Robinson et al. 1995). From there on the question became; "what is the best way to give cardioplegia?" instead of "what is the best way to protect the heart during arrest?" Cardioplegia was initially used as a cold crystalloid preparation but in the late 70's, Buckberg developed a potassium cardioplegia mixed with blood as the vehicle to improve oxygen delivery and maintain myocardial high energy phosphate compounds (Buckberg 1979). The use of blood cardioplegia became popular from the mid 80's (Robinson et al. 1995) and this gradually became the vehicle of choice for most surgeons until this day. It is interesting to observe that, despite the advantages of blood over crystalloid cardioplegia demonstrated in many small studies, some surgeons continue to use crystalloid vehicle (Jacob et al. 2008).

Component (mM)	*Bretschneider*	*Bretschneider-HTK*	*STH 1*	*STH 2*
NaCl	*12*	*15*	*144*	*110*
KCl	*10*	*9*	*20*	*16*
$MgCl_2$	-	*4*	*16*	*16*
$CaCl_2$	-	*0.015*	*2.2*	*1.2*
Procaine-HCl	*7.4*	-	*1*	-
$NaHCO_3$	-	-	-	*10*
K-ketoglutarate	-	*1*	-	-
Histidine	-	*180*	-	-
Histidine HCl	-	*18*	-	-
Tryptophan	-	*2*	-	-
Mannitol	*239*	*30*	-	-
pH	*5.5-7*	*7.1 at 25^oC 7.4at 4^o C*	*5.5-7.0*	*7.8*
Osmolality	*320*	*310*	*300*	*324*

Table 1.1 Comparison between the composition of Bretschneider and St. Thomas' Hospital solutions (Modified from Chambers (Chambers and Hearse 2001)

Recently, a large meta-analysis of blood compared to crystalloid cardioplegia was conducted; reviewing 10 randomised clinical trials and over 800 patients. This demonstrated a marginal superiority of blood cardioplegia over crystalloid cardioplegia in terms of low cardiac output syndrome and rise in cardiac enzymes. However, the incidence of perioperative myocardial infarction and death were the same (Guru et al. 2006). This marginal advantage might explain the failure of blood cardioplegia to totally dominate the practice in the same way that St. Thomas' cardioplegia did in the early 80's.

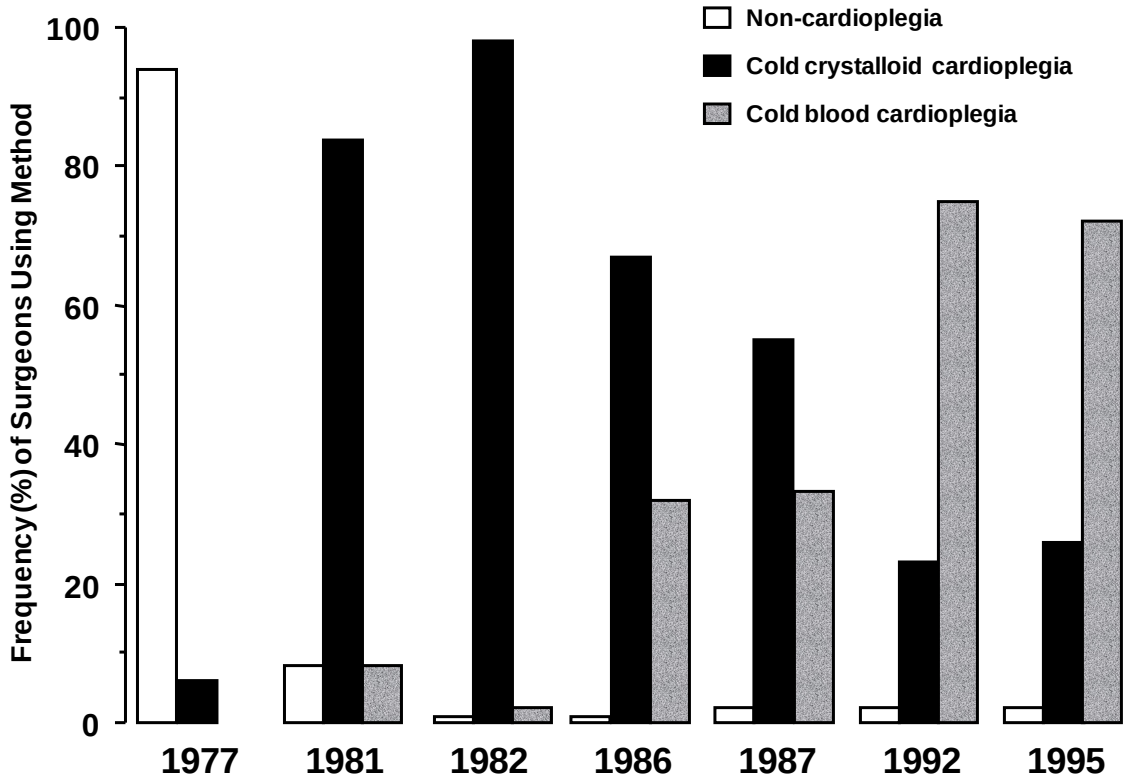


Figure 1.1. Myocardial Protection Method: Frequency in the USA (modified from Robison and colleagues (Robinson et al. 1995))

1.3 **Ischaemia and cardioplegia**

As mentioned previously, global ischaemia cannot be avoided in order to operate on a bloodless heart and in order to operate safely on the diseased heart, it is important to understand the effect of global ischaemia and try to minimise its damaging effect. Organ ischaemia can be universally defined as a deficiency in the blood supply (and thus oxygen and nutrients) to an organ to meet its physiological demand in order to function normally (Hearse DJ 1994). In the heart, the normal function is dependent on a continuous supply of high-energy phosphate compounds, particularly ATP. Approximately 60-70% of ATP is utilised by the contractile apparatus, while up to 25% is utilised by the ion translocating systems in the myocytes (Opie 1991). Since high-energy phosphate compounds are not stored in the heart, induction of ischaemia will rapidly use available ATP and this will result in gradual reduction in contractility with eventual arrest of the heart. Relatively short durations of ischaemia are associated with reversible injury, whereby early reperfusion will eventually return the myocardium to normal. However, longer periods of ischaemia induce irreversible injury associated with low levels of high-energy phosphate compounds and this will lead to cell death and myocardial necrosis (Figure 1.2). The concept of cardioplegic arrest is to prevent high-energy compound utilisation and delay the onset of irreversible injury. It is important to understand, however, that a cardioplegic solution will never abolish the effect of ischaemia totally; therefore the aim is to continue improving the cardioplegic solutions used in cardiac surgery, along with other myocardial protection strategies, to delay ischaemic injury and allow longer and more complicated surgeries to be performed without residual detrimental effect on the myocardium (Figure 1.3). Therefore, achieving reperfusion as soon as possible will always be the single most essential component of the protection process. It is also important to distinguish between regional ischaemia in the context of ischaemic heart disease and global ischaemia in cardiac surgery. Patients who are undergoing cardiac surgery for ischaemic heart disease already have an element of regional ischaemia, which could already have resulted in irreversible damage to parts of the myocardium after a myocardial infarction episode pre-operatively. Exposing these hearts to additional periods of global ischaemia while performing bypass surgery is unavoidable; the aim of cardioplegia is to delay the onset

of irreversible damage caused by global ischaemia in cardiac surgery on an already ischaemic heart with decreased functional reserve.

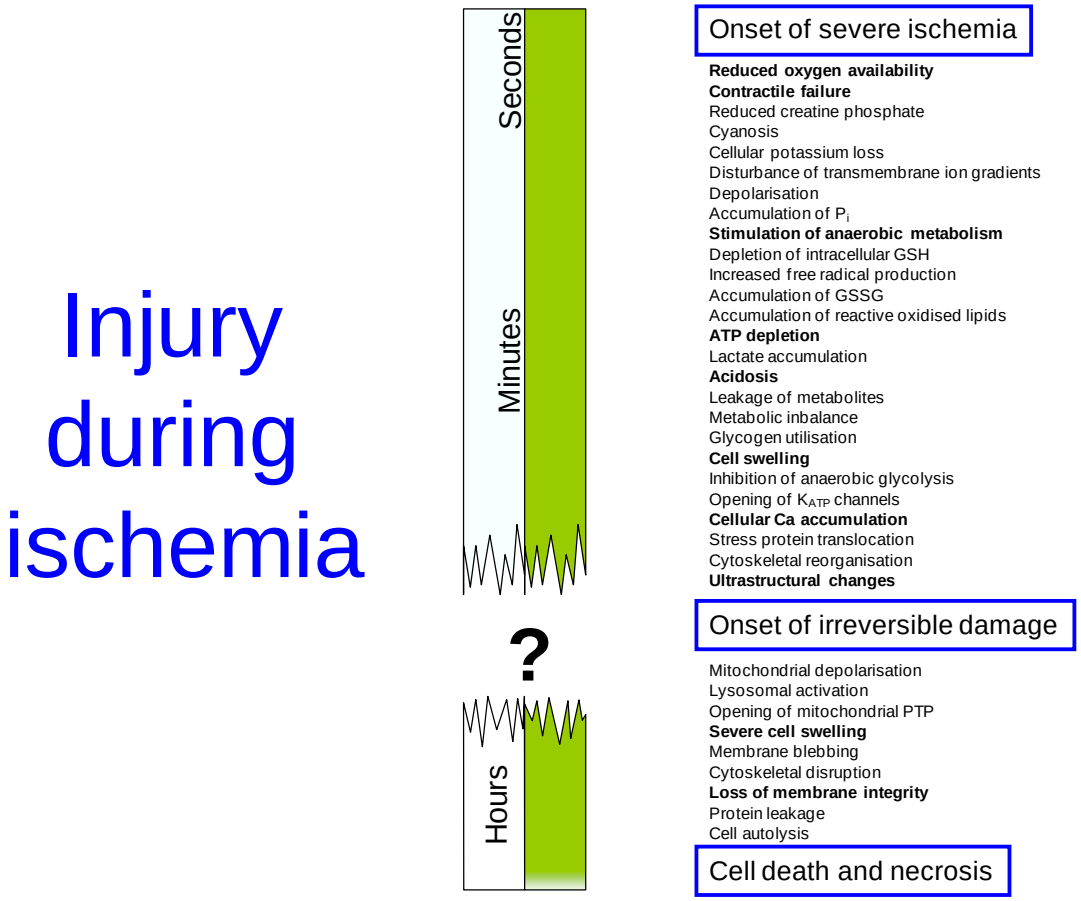


Figure 1.2 Schematic of the cellular mechanism of ischaemic injury over time (modified from Hearse and colleagues (Hearse et al. 1981))

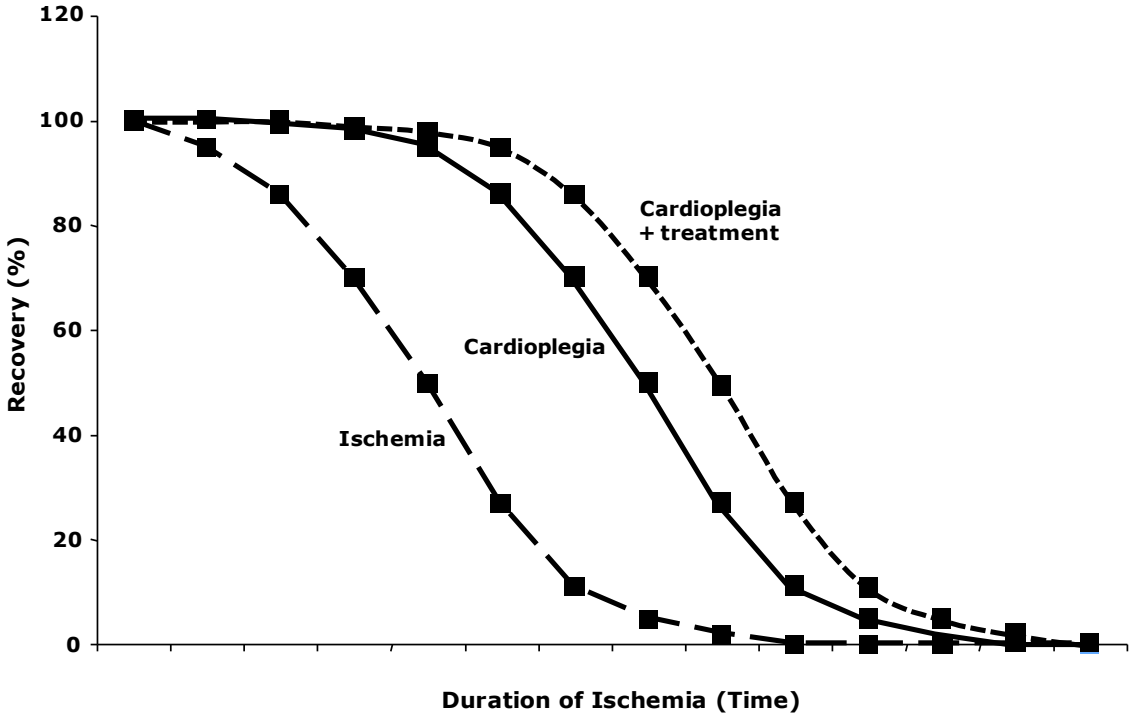


Figure 1.3 Schematic graph explaining the concept of myocardial protection associated with cardioplegia in prolonging the period of reversible ischaemia.

1.4 Pathological changes of reversible vs. irreversible ischaemia

Within seconds of the development of severe ischaemia, the myocardial cell switches from aerobic to anaerobic metabolism. This leads to reduced production of ATP and the accumulation of inorganic phosphate and lactic acid in the cell causing intracellular acidosis. Myocytes are very sensitive to inorganic phosphate and cellular acidosis and this will lead to loss of contraction within 2 minutes. Over the next few minutes, reversible ultra-structural changes occur and become visible using electron microscopy; these changes include glycogen depletion, cellular and mitochondrial swelling, myofibrillar relaxation, and margination of nuclear chromatin. If ischaemia persists (usually between 20 to 40 minutes), amorphous mitochondrial densities, sarcolemmal disruption and membrane structural changes are seen (Schoen FJ 2003). These presentations are irreversible and considered as evidence of necrosis. Figure 1.2 summarises some of the events that occurs during ischaemia. If myocardial protection is

poor, a condition described by the famous American surgeon Denton Cooley as the "stone heart" was shown to occur (Katz and Tada 1972). The stone heart, which is invariably fatal, became a challenge to the biochemists in order to find a solution. It was subsequently shown to be a result of reduced myocardial ATP content leading to irreversible ischaemic contracture caused by excessive calcium loading (Cooley DA 1972).

In summary, reversible injury to the myocytes starts very shortly after the onset of ischaemia; with more prolonged ischaemia the injury becomes irreversible. The most effective way in preventing irreversible injury is by resuming perfusion as soon as possible. However, when early reperfusion is not possible, there are other factors and interventions, which could delay this injury process and in the context of cardiac surgery, one of the most effective ways to delay this injury is to induce cardioplegic arrest. In order to understand the principles involved in inducing myocardial arrest, it is important to have detailed understanding of the physiological mechanisms involved in triggering and generating contraction in the myocardium or what is known as excitation contraction coupling.

1.5 The physiology of excitation-contraction coupling

Excitation-contraction coupling is a complex process that involves ionic changes regulated by the cell membrane in the myocardium and generating electrical changes in the cell membrane potential. These changes, which are known as the action potential are the linking switch between the excitation signal and the contractile effect. The following will briefly describe this process.

1.5.1 The membrane potential at rest

The maintenance of ionic distribution across the membrane at rest is responsible for the resting membrane potential (E_m). The difference in ionic concentrations and conductance is responsible for this value, which can be calculated using Goldman-Hodgkin-Katz (GHK) equation .

$$E_m = \frac{}{} \ln\left(P_K \frac{}{} + P_{Na} \frac{}{} + P_{Cl} \frac{}{} \right)$$

Where E_m = membrane potential

P_{ion} = permeability for the ion

$aION_o$ = extracellular activity (the concentration in free form) of the ion

$aION_i$ = intracellular activity of the ion.

R = gas constant

T = temperature in kelvins

F = Faraday's constant

z = valency of the ion

The advantage of the GHK equation is that it can calculate the membrane potential when there is more than one permeable ion, which is very relevant to the cardiac myocyte. While the more simplistic Nernst equation calculates the E_m in a model when a membrane is impermeable to one ion, which is potassium in this case; therefore it is assuming that the membrane is completely permeable to the rest of the other ionic species (Katz 1992).

$$E_m = \frac{}{} \ln\left(P_K \frac{aK_o}{} \right)$$

From the Nernst equation, we can extrapolate that for an alkaline ion like Na^+ and K^+, z=1 and a membrane fully permeable to the ion, p=1, at 37^{o}C the equation can be simplified to

$$E_m = 61.5 \ln\left(\frac{aK_o}{} \right)$$

In the resting cardiac myocyte where $[K^+]_o$ is ~ 5.4 mM and the $[K^+]_i$ is ~ 110 mM, the potassium activity is about 4 and 100 mM respectively. E_m therefore for a membrane that is fully permeable to K^+ at these activities is E_m=61.5 log (4/100) = -86 mV. At rest the membrane potential is close to -80 mV, which suggests that the permeability to potassium at these concentrations is near 1 (fully permeable) and possibly less permeable to other ions. Intracellularly, K^+ is the principal cation, and phosphate and the conjugate bases of organic acids are the dominant anions. While outside the cell, Na^+ and Cl^- predominate. It is important, however, to bear in mind that the cell membrane is not a simple semi-permeable membrane (Katz 1992); the ionic permeability of this membrane is determined by sophisticated mechanisms, which involves the activity of certain ionic channels, pumps and other membrane exchanging mechanisms like the Na^+/K^+ pump, the Na^+/Ca^{2+} exchanger current and the I_{Kr} inwardly rectifying K^+ current (Bers 1991).

The resting period in the cardiac cycle is responsible under physiological conditions for the diastolic ventricular phase, which allows the heart to fill with blood, ready to eject during the next contraction. This phase is called phase 4 during the membrane action potential (Figure 1.4) and is particularly important in cardiac surgery because the heart is arrested during this phase for a long period. Furthermore, the membrane potential during cardioplegic arrest is one of the factors that determine the effectiveness of an arresting agent in myocardial protection as explained in detail later.

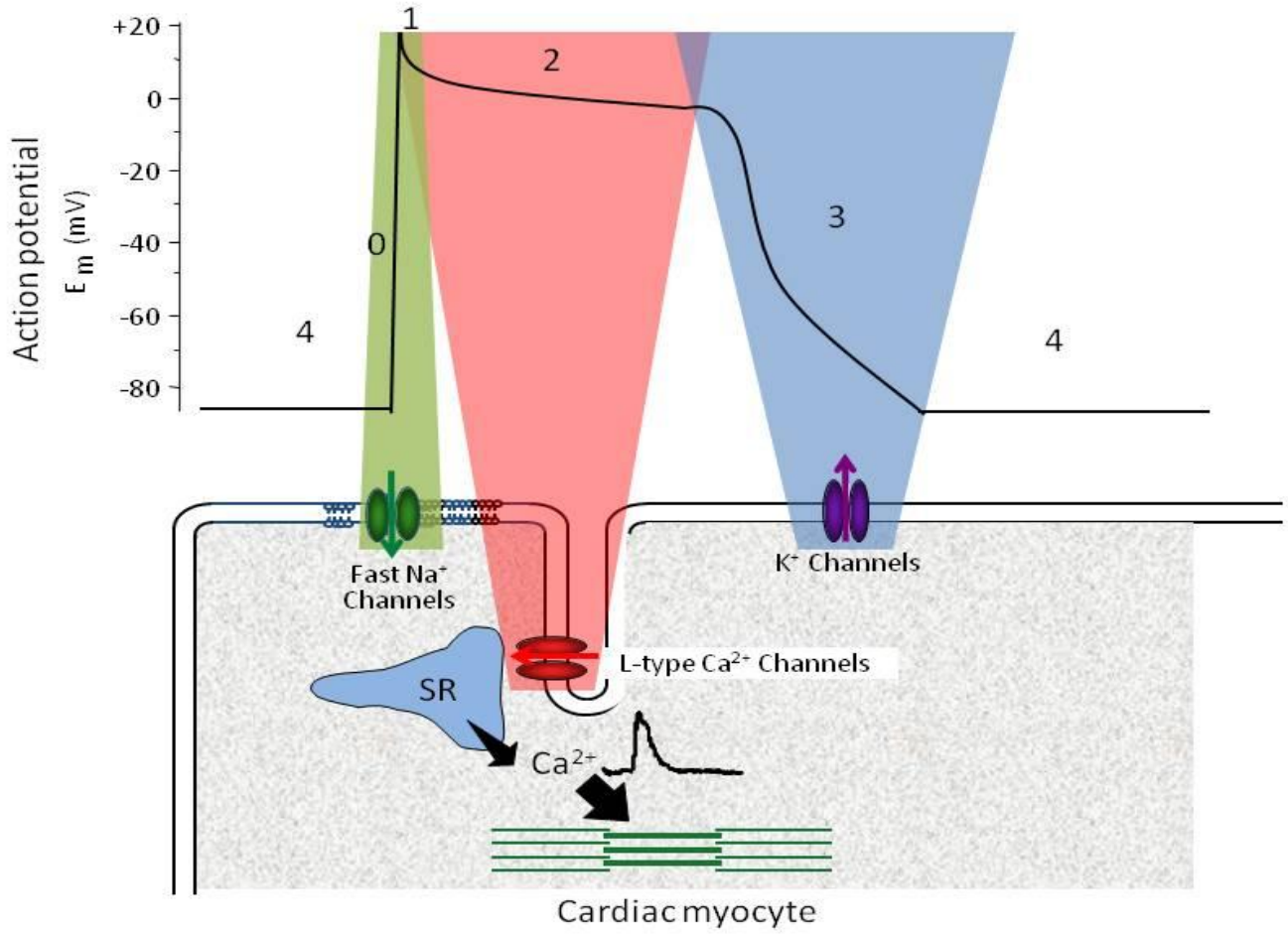


Figure 1.4 Schematic of cardiac myocyte and the membrane action potential.

1.5.2 The rapid depolarisation phase

During excitation and the changes in the action potential the permeability of the membrane to the various ionic species is constantly changing due to the activation of various channels, pumps and exchangers. Therefore, the Nernst equation, which takes into account a single ion and single permeability, becomes inadequate to calculate the E_m. The GHK equation, despite taking into account more than one ionic species, also has its limitations during the action potential as it calculates E_m in the steady state without any changes in the permeability of the membrane via the mechanism mentioned earlier (Katz 1992).

At the resting E_m all the fast Na^+ channels are closed but excitable to electrical stimulation to the membrane by the conducting system or by a pacemaker. This results in immediate and simultaneous opening of these channels, allowing a rapid entry to Na^+ inside the myocyte and making the membrane more permeable to Na^+(Niedergerke and Orkand 1966). As a result, the E_m will change due to the changes in the sodium permeability (P_{Na^+}), $[Na^+]_o$ and $[Na^+]_i$ according GHK equation (above) and the membrane polarity changes to +20mV (close to Na^+ equilibrium potential of around +50mV). This phase is called phase 0 (Figure 1.4) and is responsible for initiating contraction in the cardiac cycle. After the peak of phase 0 there is a small downward deflection of the action potential due to the movements of K^+ and Cl^+ and this is called phase 1 (Figure 1.4) (Opie 1991; Katz 1992).

The fast Na^+ channels are double-gated according to the Hodgkin-Huxley model. They are complex proteins embedded in the cell membrane and are formed of two types of protein subunits, α and β (Hodgkin et al. 1952; Katz 1992). The α-subunit assembles to form the functional core of the channel. The α-subunit protein is sufficient to enable the cell to conduct Na^+ in a voltage-gated way, without the β-subunits. The β-subunits are responsible for the channels altered voltage dependence and cellular localisation (Katz 1992). The α-subunit (Figure 1.5, A,B) has four repeated domains I-IV, each containing six membrane-spanning regions, labelled 1-6. Region 4 acts as the channel's voltage sensor (Katz 1992). The high voltage sensitivity of the channel is due to the strictly

conserved amino acid sequence in this sensing region with the positively charged amino acid arginine and lysine around the membrane embedded part. This arrangement enables the sensor to move within the membrane bilayer as membrane potential changes (Katz 1992). The Na^+ channels structure is an example of a voltage-dependent channel described by the Hodgkin and Huxley principle (Hodgkin et al. 1952). The function and the state of these channels are determined by the position of two gates; the activation and inactivation gates (Figure 1.5, B and C). During the propagation of the action potential the sensing region is stimulated by a change in membrane voltage. This region moves toward the extracellular side of the cell membrane, displacing the activation gate (region 6) and opening the pore of the channel. This leads to further depolarisation of the membrane potential, which opens more activation gates at a very fast rate (0.1-0.2 ms) (Niedergerke and Orkand 1966), closer to the Na^+ equilibrium potential of +50 mV (Katz 1992). As the membrane potential depolarises, over 1 ms later, the inactivation gate formed by links between domains III and IV closes by forming a tethered plug to the intracellular side of the channel gap, thereby terminating the I_{Na} current. The channels then remain refractory in the inactive state until these inactivation gates reopen much later in the action potential when repolarisation is restored. At membrane potentials between -20 and -60 mV the two gates can become partially opened at the same time allowing constant slow entry of the Na^+ inside the cell, generating what is known as the Na^+-window current.

The selectivity of the channel to Na^+ is due to the negatively charged amino acid sequence in the extracellular part of the entry pore, which is narrowed down (0.3-0.5 nm) by the selectivity filter in regions V and VI. This amino acid sequence attracts the positively charged ions and keeps the negatively charged ions out, allowing only one Na^+ ion linked with a water molecule in this narrow pore. The larger K^+ and Ca^{2+} ions cannot fit and enter this channel. Also, other smaller size cations do not interact well with the negatively charged glutamic acid lining the pore (Opie 1991). Agents that inactivate these channels or block their pores can induce arrest and hence can be used as cardioplegic agents.

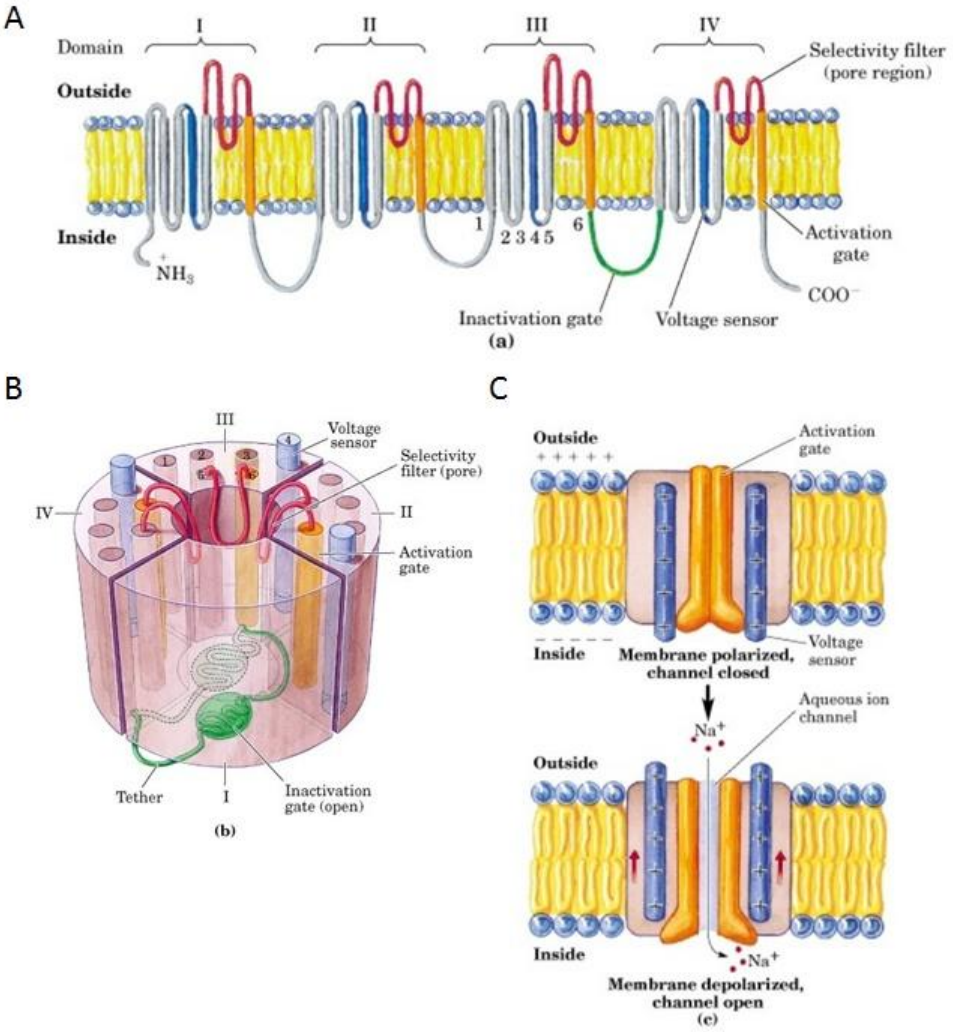


Figure 1.5 the structure of the alpha subunit of fast voltage gated Na^+-channels; A, the linear structure; B, the three dimensional structure; C, the channel opened and closed (modified from http://courses.cm.utexas.edu/jrobertus/ch339k/overheads-2/ch12_volt-gated-chan.jpg)

1.5.3 The plateau phase

During the plateau phase (phase 2), the action potential stabilises despite many currents being activated, such as the outward K^+ current via the slow delayed potassium channels, the Na^+/Ca^{2+} exchanger current, and others. This is because these currents are balanced by an inward current of Ca^{2+} through the L-type calcium channels. This current makes the cardiac action potential unique and is responsible for the refractory period of the cardiac myocyte, which ensures effective contraction and prevents arrhythmias. Additionally, it provides the transient rise in intracellular Ca^{2+} concentration essential for contraction (Bers 1991). Inhibitors of these channels can inhibit contraction and therefore can be used as cardioplegic agents (Yamamoto et al. 1983). The mechanism of calcium channels in relation to cardiac arrest requires a detailed description of their mechanism of action. Therefore the calcium channels have relevant importance and described in details next.

The effect of Ca^+-channels was discovered accidentally in the Biophysics lab at the University College London in 1952 by Bernard Katz and colleagues (Fatt and Katz 1953). While investigating the membrane potential using voltage clamping studies in large muscle cells of crab and crayfish; they, unexpectedly, found electrical responses that persisted in sodium-free media, when Na^+ was replaced by tetrabutylammonium (TBA). One speculative of mechanism to explain this current was a calcium-mediated or magnesium-mediated inward current. In the heart, the plateau phase was found to be more sensitive to divalent ions such as Ca^{2+} and Mg^{2+} (Wray et al. 2004). This current was attributed to what was mistakenly called the dihydropyridine receptors (DHPRs) after the calcium antagonist nifedipine (Dolphin 2006). These were later shown to be voltage channels to Ca^{2+} and the cardiac types were identified as the L-type and the less significant T-type (Bers 2002). The L-type channels are voltage-gated pores localised in the cell membrane. They become activated at E_m between -50 and -10 mV allowing Ca^{2+} inside the cell according to the Hodgkin and Huxley principle (Hodgkin et al. 1952). The biochemical structure is formed of a complex protein structure with four or five subunits. The $\alpha 1$ subunit of 190-250 Kilo Dalton is the largest subunit and it incorporates a conduction pore similar to that of the sodium channels; it contains a

voltage sensor and gating apparatus. The α1-subunit of voltage-gated calcium channels is organised into four homologous domains (I-IV) with six transmembrane regions in each. As in sodium channels, the fourth region is the voltage sensor and the selectivity filter is formed by the pore loop between regions 5 and 6. The selectivity of the channel to Ca^{2+} is due to changes of only three amino acids in the pore loops in domains I, III, and IV from the sequence in the sodium channels. An intracellular β-subunit and a transmembrane, disulphide-linked α2δ-subunit complex are components of most types of calcium channels. A γ-subunit was also found in skeletal muscle calcium channels and related subunits are expressed in heart and brain. Although these auxiliary subunits modulate the properties of the channel complex, the pharmacological and electrophysiological diversity of calcium channels arises primarily from the existence of multiple α1-subunits (Opie 1991; Catterall 2000).

1.5.4 The repolarisation phase

During phase 3 of the action potential (Figure 1.4), the calcium channel current ($I_{Ca,L}$) declines due to the inactivation of the channels and an ongoing outward K^+ current via the slow delayed rectifier (I_{Ks}). This shifts the E_m to more negative values, activating other K^+ channels like the rapid delayed rectifier. These channels are sensitive to ATP and are referred to as the ATP-sensitive potassium (K_{ATP})-channels (Opie 1991). Activating K_{ATP}-channels pharmacologically will result in shifting the resting E_m closer to the K^+ equilibrium potential, leading to hyperpolarisation. This makes the sodium channels less excitable and could result in cardiac arrest. K_{ATP}-channel activators, like pinacidil, have been used through this mechanism to induce cardioplegic arrest using (Jayawant and Damiano 1998). Due to the importance of these channels in inducing cardioplegic arrest they are described in detail below.

(K_{ATP}) channels were first described by Noma in the myocardium (Noma 1983). When they are activated they generate a small K^+ outward current to balance the inward current of Ca^{2+} and Na^+. Eventually, the L- type calcium channels become inactivated and repolarisation is re-established with this polarising current. The K_{ATP}-channels are

inhibited by the physiological intracellular [ATP] of around 3-4 mM (Khairallah and Mommaerts 1953). When [ATP] drops below 1 mM these channels open allowing high efflux of K^+. At rest the sarcolemma is a semi-permeable membrane to K^+, shifting the resting membrane potential closer to K^+ reversal potential of -90mV. However, due to a small inward leak current of Na^+, this potential is offset by +10 mV to -80mV, which is the physiological resting membrane potential. The activation of K_{ATP}-channels by the K_{ATP} openers generates an outward K^+ current offsetting the small Na^+ current and hyperpolarising the membrane closer to -90mV. An arrest induced by these agents is called hyperpolarised arrest, which has its advantages in the use of cardioplegia.

1.5.5 Calcium-induced calcium-release

The rise in intracellular [Ca^{2+}] during the plateau phase of the action potential leads to activation of the ryanodine receptors (RyR) due to the close localisation of these channels in the dyadic junction in the T-tubules of the sarcolemma (Bers 2002). RyR activation releases significant amounts of Ca^{2+} from the sarcoplasmic reticulum (SR); and this process is known as calcium-induced calcium-release. The sensitivity of the RyR to Ca^{2+} on its cytosolic side is responsible for the positive feedback mechanism in RyR activation, and explains the fast non-linear rise in the intracellular calcium transient (Ca_{tr}) (Bers 1991). A cluster of RyR in the cytosol is activated in response to localised increase in [Ca^{2+}] resulting in a spatiotemporally-restricted rise in cytosolic Ca^{2+} that can be visualised as a "calcium spark" (Cheng et al. 1993). There are different isoforms of the RyR; RyR_1 is primarily expressed in skeletal muscle, RyR_2 in the myocardium, RyR_3 is widely expressed but mainly in the brain and there is RyR_4 expressed only in fish (Zucchi and Ronca-Testoni 1997). The function of the SR is to magnify the intracellular rise of calcium as a response to the activation of the L-type calcium channels creating Ca_{tr}. This results in more cytosolic Ca^{2+} available for the myofilaments to induce contraction as a response to the excitation stimulus.

1.5.6 Cross-bridging and myofilaments contraction

The interaction between the thick (myosin) heads and thin (actin) myofilaments is known as crossbridging and is translated into shortening in the myocytes. These myofilaments are in close contact with the sarcolemma, which invaginates at the levels of the Z-lines forming the T-tubules (Figure 1.6, A). Myosin heads binding to actin is regulated by the crossbridge regulatory proteins; the first is troponin (Tn) with the three subunits C, I and T, and the other protein is tropomyosin, which separates the actin from the myosin heads and resides in the cleft between the two actin strands (Figure 1.6, B). When the intracellular ionised Ca^{2+} rises during the Ca^{2+} -induced Ca^{2+} -release phase, TnC binds to Ca^{2+} inducing conformational changes to the troponin complex and causing TnI to dissociate from actin. Tropomyosin then becomes dissociated from the actin cleft revealing myosin binding sites. These sites allow the crossbridges to form between the myofilaments, resulting in tension and shortening of myocytes (Solaro and Rarick 1998).

In summary, myocardial contraction is a staged process involving a complex interplay between ionic changes occurring during the action potential and the contractile mechanism of the sarcomere. This staged process enables us to understand the various mechanisms of contraction and by, targeting these stages and mechanisms pharmacologically we can induce effective cardioplegic arrest. We aim next to define cardioplegia and systematically review the mechanisms of various cardioplegic solutions available experimentally and clinically based on the stages of excitation contraction-coupling.

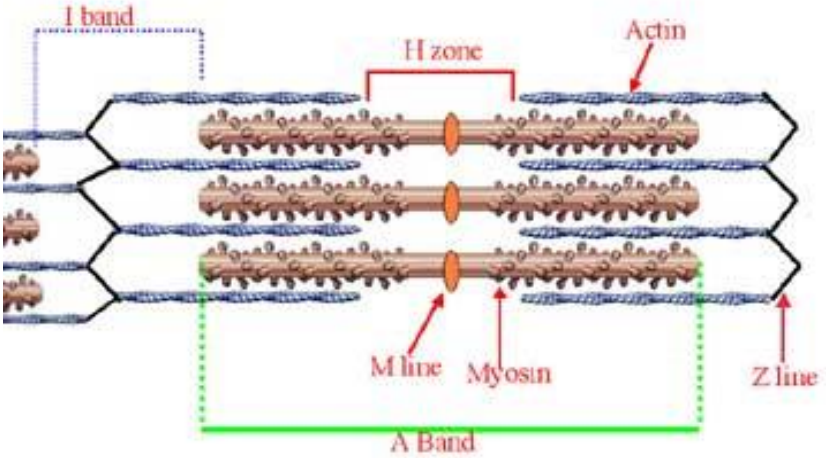
1.6 **Cardioplegia**

The term “cardioplegia” was first used in 1957 to define the elective rapid and reversible chemical arrest of the heart in order to perform cardiac surgery (Lam et al. 1957). However, cardioplegia should also aim to offer protection to the heart against ischaemia among other things addressed below.

1.6.1 The aims of cardioplegia

The aim of using cardioplegia in cardiac surgery is to allow the surgeon to operate on a quiescent flaccid empty heart and protect it from the consequences of global ischaemia and reperfusion caused by aortic cross clamping to provide a bloodless surgical field. Although there are many agents capable of inducing cardiac arrest, an effective cardioplegic arrest solution should be able to meet the following criteria:

A



B

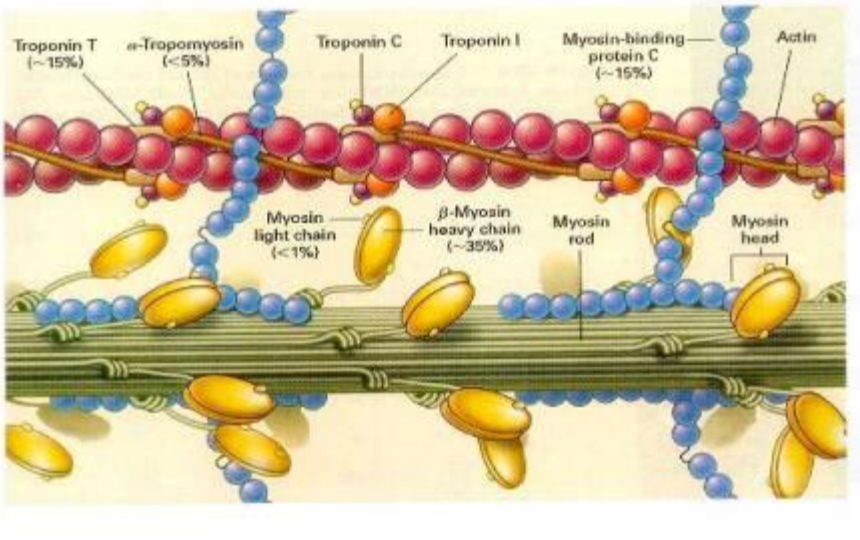


Figure 1.6 The sarcomere structure, A, a schematic of a sarcomere, B, the interaction between the actine and myosin filaments. Modified from http://www.edcenter.sdsu.edu/cso/paper/image005.jpg

I. **Arrest:** A rapid and effective induction of diastolic arrest to keep the myocardium relaxed and to minimise cellular use of ATP.

II. **Myocardial protection:** Protective effects to delay the onset of irreversible injury caused by global ischaemia and to limit the extent of reperfusion injury.

III. **Reversibility**: Readily-reversible cardioplegic effects on washout for quick resumption of heart function, to enable early weaning off cardiopulmonary bypass.

IV. **Low toxicity:** A short half-life with no toxic effects on other organs after cessation of cardiopulmonary bypass. This is especially important if high concentrations of the pharmacological agent are required to achieve arrest.

1.6.2 Variation of cardioplegic solutions

Cardioplegic solutions have been widely used for more than 30 years. This has triggered considerable research work to establish the optimal cardioplegic solution and the best way to introduce it into the heart. Although hyperkalaemic cardioplegia remains by far the main concept used, the ongoing work to establish the best way to introduce it resulted in different preparations and methods of delivery. The following list is aimed to include all solutions used clinically or experimentally based on the following criteria**.**

1 **Vehicle:** cardioplegia can be given as a crystalloid (asanguineous) solution (Chambers and Hearse 2001) or autologous blood (Buckberg 1979).

2 **Temperature:** This can be cold (4°C), patient's temperature (35-37°C), Tepid (29°C), room temperature (23-25°C), or as a "hot shot" at the end, known as terminal cardioplegia. Although the term "hot shot" can vary in practice from blood based high K^+ cardioplegia at higher temperature (37^oC) to a warm blood solution with added substrate the principle is to give it as a treatment after ischaemia near the end of the cross-clamp period, just before reperfusion (Imura and Suleiman 2008). The aim is to restore the cellular high energy compounds before contraction. This strategy was shown to have advantages over standard cold blood cardioplegia during

ischaemia only in patients undergoing CABG (Caputo et al. 1998). The same group, however, demonstrated that these findings can vary between the chronic ischaemic and the hypertrophied myocardium. Ascione and colleagues demonstrated that there is no clear advantage in adding a "hot-shot" in patients with hypertrophied left ventricle undergoing aortic valve surgery (Ascione et al. 2008).

3 **Methods of delivery:** The most widely used method of delivering cardioplegia is through an antegrade perfusion via a cannula inserted into the aortic root, down the coronary arteries. This, however, becomes difficult in aortic regurgitation as the solution could leak into the LV cavity. To overcome this problem, retrograde perfusion can be used. It was first described in 1956 (Lillehei et al. 1956) and it is administered via a cannula in the coronary sinus through the coronary veins back into the coronary arteries. Other potential advantages of retrograde perfusion include, in principle, better delivery to the territories supplied by occluded coronary arteries in ischaemic heart disease but there is no convincing evidence in the literature to support this (Allen et al. 1995). Retrograde cardioplegia can also be administered easily without getting in the way of the surgeon, especially in the context of mitral valve surgery where antegrade cardioplegia requires repositioning of the heart and interrupting the surgery during each administration. The main disadvantages of retrograde cardioplegia however include its poor perfusion to the right ventricle and therefore it offers suboptimal right ventricular protection (Allen et al. 1995). Another disadvantage is the technical difficulty in introducing the cannula via the right atrium in the coronary sinus and the potential injury to the coronary sinus.

4 **Infusion interval:** Cardioplegia is usually given intermittently but occasionally it can also be given continuously, usually retrogradely.

5 **The mode of arrest:** The above criteria address the variable methods of introducing the cardioplegic solution into the patient and have been mainly used with hyperkalaemic cardioplegia. Considerable clinical and experimental research has been conducted in order to establish the best way to administer hyperkalaemic cardioplegia, in terms of temperature, vehicle and methods of perfusion. This has

generated a large volume of literature, which can, sometimes, be confusing (Jacob et al. 2008); however, this multitude of studies does suggest that improved cardioplegia is still required. The advantages of simply further modifying the current techniques of hyperkalaemic cardioplegia are likely to be marginal. Thus, new concepts of arrest that may provide potentially beneficial alternatives to hyperkalaemia are required, for any chance of achieving significant improvement over the widely accepted hyperkalaemic arrest (Fallouh and Chambers 2008). The following paragraph will discuss the potential modes of action of arresting agents in detail and try to describe the disadvantages and disadvantages of each.

1.7 **Mechanism of inducing cardioplegic arrest**

In order to induce chemical arrest, the arresting agent should be aimed at cellular targets in the cardiac myocytes or the conduction tissue, which takes part in the excitation contraction coupling. This can be summarised into the following groups (Figure 1.7)

a) Inhibiting the fast sodium current (I_{Na}) to prevent the propagation of the myocardial action potential.
   - Extracellular hyperkalaemia (eg. STH)
   - Sodium channel blockers (lidocaine, procaine, tetrodotoxin (TTX))
   - K_{ATP}-channel openers (pinacidil, adenosine)

b) Inhibiting calcium activation of the myofilaments to prevent myocyte contraction.
   - Zero extracellular calcium (Bretschneider solution (Bretschneider et al. 1975)
   - L-type calcium-channel blockers (high magnesium, diltiazem, verapamil)

c) Direct myofilament inhibition (myofilament Ca^{2+} desensitisation with 2,3-butanedione monoxide (BDM)

1.7.1 Inhibiting the fast sodium current

1.7.1.1 Hyperkalaemia

Elevation of the extracellular potassium concentration ($[K^+]_e$) to 10-40 mM will shift E_m from about -85 mV to a range between -65 to -40 mV, according to the GHK constant field equation. At potentials above -65mV, most of the fast sodium channels that are responsible for phase 0 of the action potential become inactive. At higher $[K^+]_e$, leading to E_m over -40 mV, the L-type calcium channels are activated, which will lead to inward calcium flux and calcium loading. Therefore, it is important to use $[K^+]_e$ within the range that inactivates the fast sodium channels without activating the L-type calcium channels. Hyperkalaemia induces arrest by shifting the resting E_m away from the physiological polarisation voltages; thus it was termed as depolarised arrest. In order to determine the optimal $[K^+]$ to induce arrest in the rat hearts, Hearse and colleagues found that $[K^+]$ concentrations between 16 to 20 mM, which could induce arrest without inducing too high coronary resistance and low coronary flow. (Figure 1.8) (Hearse et al. 1975). This was, interestingly, consistent with the values extrapolated from the GKH equation (Figure 1.9).

Gay and colleagues used much higher $[K^+]$ of 40 mM in their hyperkalaemic cardioplegia (Gay and Ebert 1973) earlier. Thereafter, numerous studies by Hearse and colleagues were conducted to further improve hyperkalaemic cardioplegia by optimising temperature (Hearse et al. 1975), mode of delivery and the additives to high potassium (Hearse et al. 1976). Additional examples explored the value of additives (Mg^{2+} (Hearse et al. 1978), high energy phosphate compounds (Robinson et al. 1984) (Robinson et al. 1987) and procaine (Hearse et al. 1981)) to cardioplegia; in "working mode" perfused rat hearts, subjected to 30 minutes of normothermic ischaemia, these compounds improved recovery of aortic flow from around 30% for hyperkalaemia only to over 93% with cumulative effect of Mg^{2+}, ATP, procaine and creatine phosphate (Hearse et al. 1976). Ultimately the extensive characterisation strategies to optimise cardioplegia paired with the clinical insight and the need to deliver an effective solution resulted in the development of the St. Thomas' Hospital cardioplegic solution No.1

(STH1) in 1975. This solution was designed to achieve a balance between simplicity and maximum myocardial protection (Hearse et al. 1981). STH1 contains Mg^{2+} and procaine (but did not contain other agents like ATP and creatine phosphate) and did not have any buffering capacity for the purpose of simplicity (Hearse et al. 1981). Further work and development were made on STH1 solution, which resulted in the development of the St. Thomas' Hospital cardioplegic solution No.2 (STH2) (Jynge et al. 1981). In STH2, $[K^+]$ was decreased from 20 to 16 mM, $[Ca^{2+}]$ was also reduced to 1.2mM, a buffer was added in the form of $NaHCO_3$ and procaine was removed to enable the solution to be registered with the FDA, which does not recognise procaine for human use (Table 1.1). Depolarised arrest by STH1 and STH2 is proven to be an effective, safe and reliable means of arresting the heart during heart surgery, explaining its wide use. However, shifting E_m to voltages close to -50mV makes the fraction of activated and inactivated I_{Na} channels equal in the steady state. This generates an inward window current of Na^+ (at E_m between -60 and -15mV) during the period of ischaemia and depolarisation. Ischaemia can further increase the Na^+ entry by activating the Na^+/H^+ exchanger (driven by intracellular acidosis (Pike et al. 1993; Satoh et al. 1995)) and the inactivation of Na^+/K^+-ATPase (assisted by hypothermia (Lahorra et al. 1997)). As a result the myocyte becomes loaded with Na^+, which causes increased osmolality and cell oedema. At reperfusion, the high intracellular Na^+ drives the Na^+/Ca^{2+} exchanger, which plays a major role in heart failure patient (Terracciano et al. 2007), becomes active in reverse mode and the Na^+ is exchanged with Ca^{2+} (Satoh et al. 1995; Satoh et al. 2000), which lead to Ca^{2+} loading. Higher $[K^+]$ like the Gay solution (Gay and Ebert 1973) can potentially activate $I_{Ca,L}$ window currents and worsen Ca^{2+} loading. Reperfusion Ca^{2+} loading can trigger contracture and cell death (Figure 1.10).

Na^+ and Ca^{2+} loading are features of ischaemia and although hyperkalaemia might, it can result, in Na^+ loading (Schepkin et al. 1999). This is possibly by the effect of preventing prolonged ischaemia and reinstating perfusion as soon as possible.

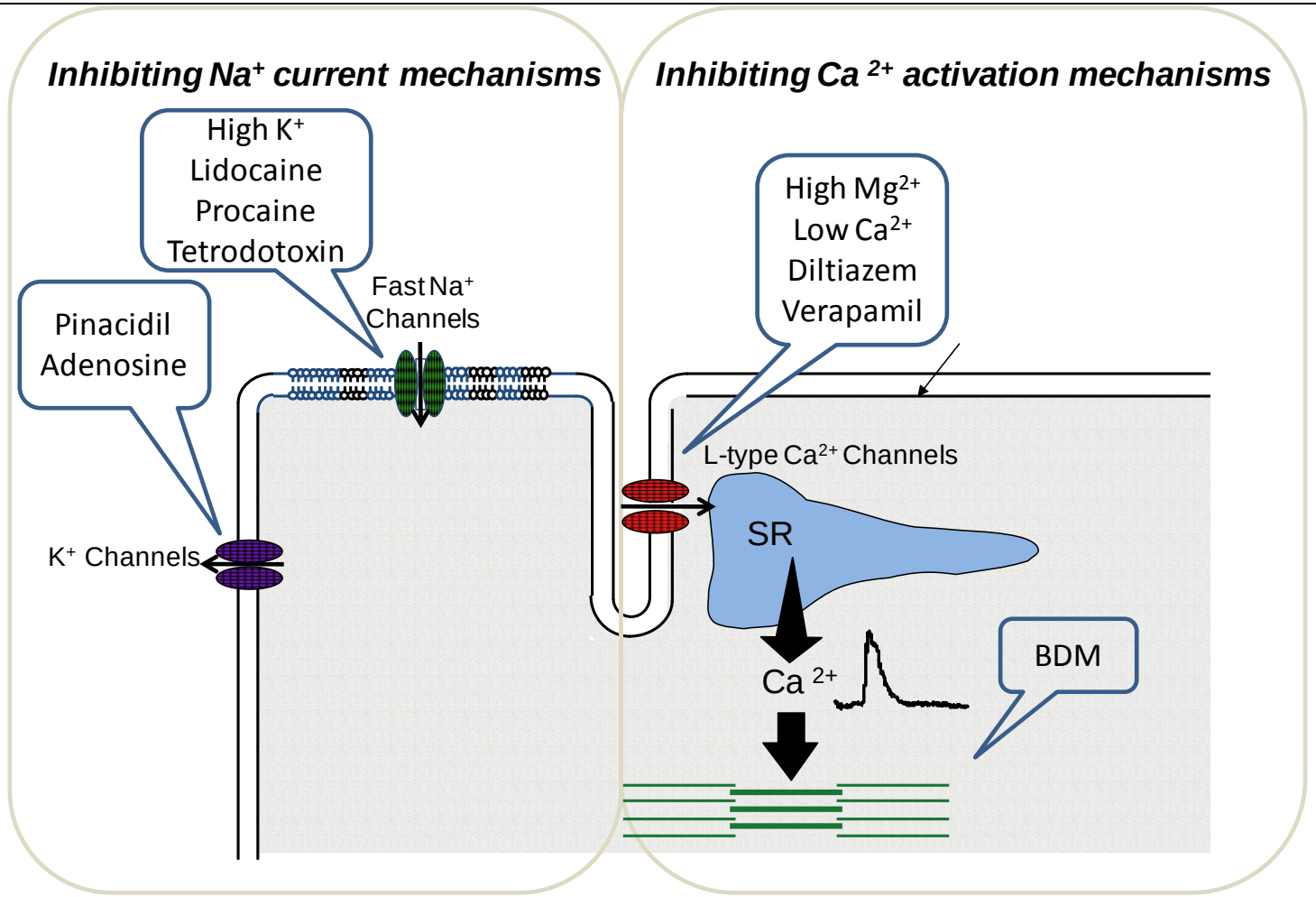


Figure 1.7 The cellular targets for cardioplegic arrest and examples of pharmacological arresting agents. Figure modified from (Fallouh et al. 2009)

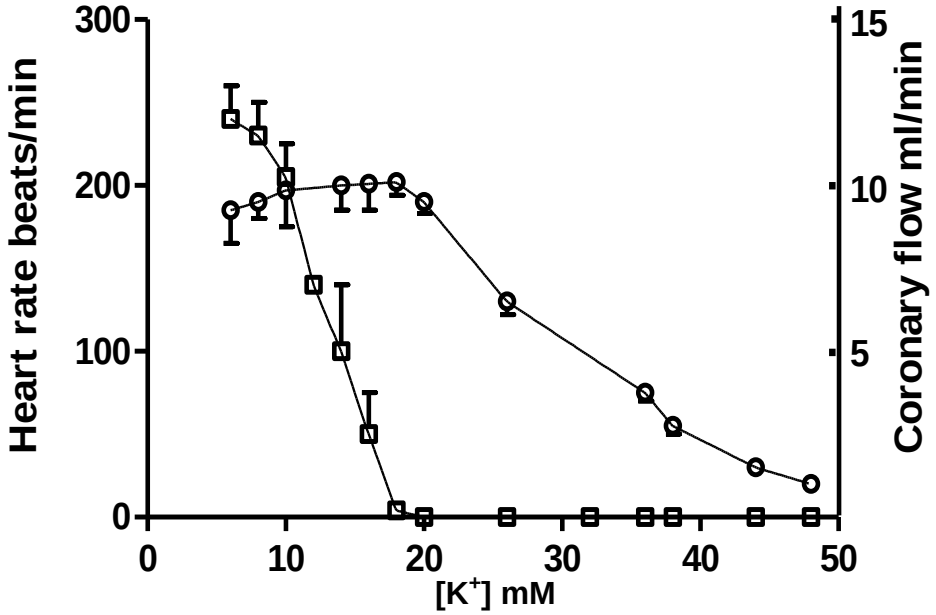


Figure 1.8 The relationship between [K^+] and heart rate (squares) and coronary flow (circles), in the isolated rat hearts, data presented as Mean ± SEM. (Modified from (Hearse et al. 1981))

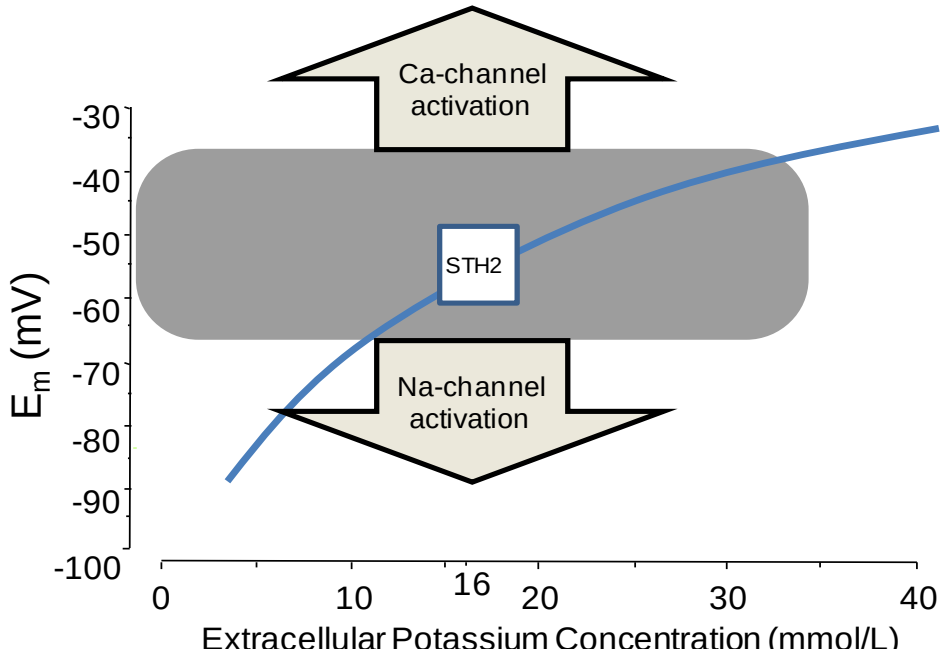


Figure 1.9 Cell membrane potential in relation to extra-cellular potassium (modified from (Bers 1991))

1.7.1.2 Na^+ Channel blockers

I_{Na} blockers like lidocaine can arrest the heart by inhibiting the action potential at phase 0 and has proven to be an effective arresting agent (Asano et al. 2003; Dobson and Jones 2004; Yamaguchi et al. 2007). Additional protection was also demonstrated in a long term preservation with procaine when it was added to hyperkalaemic cardioplegia (Hearse et al. 1981) and consequently was included in STH1 (Table 1.1). Blocking the I_{Na} current with these agents maintains E_m close to the resting potential, inducing a "polarised arrest". This was demonstrated in a long-term preservation study by Snabaitis and colleagues (Snabaitis et al. 1997) in which rat hearts were stored for 5 hours at 7.5^{o}C and E_m was measured throughout ischaemic storage using sharp electrode on the surface of the heart. Three groups of rat hearts were arrested using hyperkalaemia (16 mM), the Na-channel blocker tetrodotoxin (TTX) or Krebs-Henseleit Buffer (KHB) then were stored at 7.5^{o}C for 5 hours. E_m was measured during the 5 hour cold ischaemia period. E_m in the hyperkalaemia group dropped instantaneously to -50mV and remained throughout the period of ischaemia while the TTX group remained at around -70mV. The KHB group started at -70 mV and depolarised during ischaemia to -50mV (Figure 1.11). At membrane potentials close to -70 mV, the Na^+ 'window' current is absent (McAllister et al. 1975; Attwell et al. 1979).

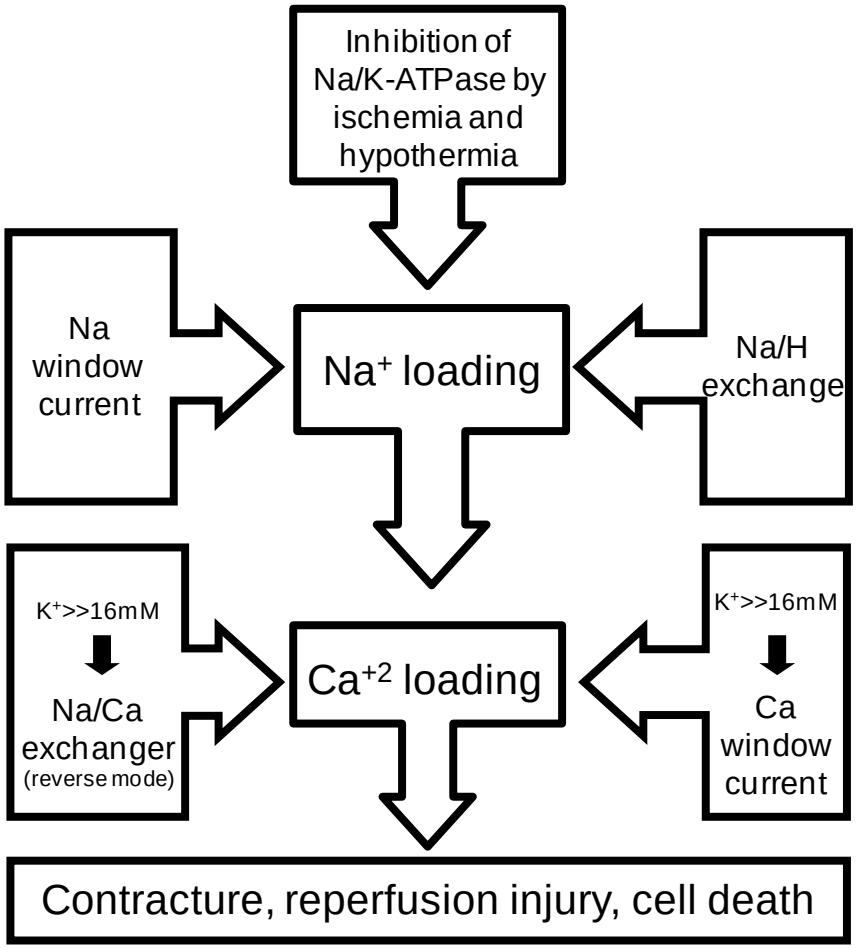


Figure 1.10 The mechanism of Na^+ and Ca^{2+} loading during depolarised cardioplegia and global ischaemia

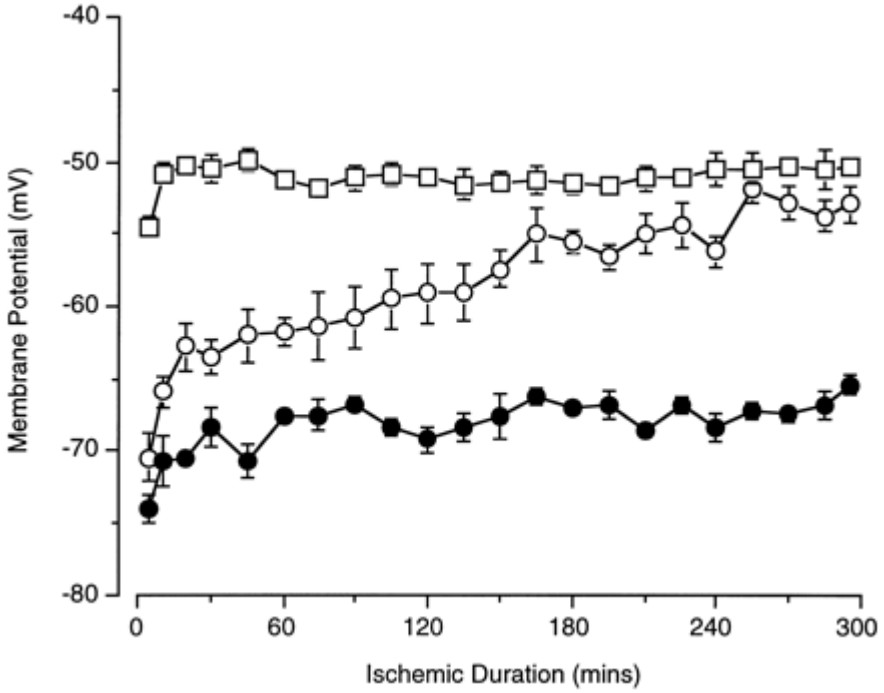


Figure 1.11 Cardiac E_m recorded every 15 minutes during 5 hours of hypothermic (7.5°C) ischemic arrest with KHB(○), hyperkalaemic arrest (□), and TTX arrest (▪). (figure modified from Snabitis and colleagues (Snabaitis et al. 1997))

Van Emous and colleagues (Van Emous et al. 1997) investigated the role of the fast sodium channels in sodium loading using nuclear magnetic resonance (NMR) studies. They demonstrated that in hearts pre-treated with lidocaine, the intracellular Na^+ concentration rise during normothermic ischaemia (30 min) was inhibited compared with global ischaemia alone. Myocardial ATP levels were higher and recovery of developed and end-diastolic pressure with lidocaine. They concluded that the fast sodium channels are involved in the Na^+ loading during ischaemia and that blocking these channels pharmacologically could inhibit Na^+ loading. On the other hand, Schepkin and colleagues (Schepkin et al. 1996; Schepkin et al. 1999) demonstrated, using NMR, that the fast Na^+ loading, which takes place during ischaemia and can only be partially reduced in depolarised cardioplegic arrest; single dose hyperkalaemic cardioplegia followed by normothermic ischaemia in the Langendorff rat heart resulted in onset of increased $[Na^+]_i$ after 20 min and after 51 min there was a significant rise in $[Na^+]_i$ but this remained significantly less in than the ischaemia alone group. However,

multidose hyperkalaemic cardioplegia at 17 minutes interval resulted in very little Na^+ loading after 51 minutes of normothermic ischaemia in the rat heart (Schepkin et al. 1999).

It is difficult to be certain that the mechanism behind any improved protection against ischaemia offered by Na^+ channel blockade over the traditional hyperkalaemic cardioplegia is by inhibition of Na^+ loading as we are not aware of any NMR studies that directly compares polarised cardioplegic arrest by Na^+ channel blockade with depolarised arrest by hyperkalaemia. However, it is reasonable to assume that direct Na^+ blockade would offer a feasible alternative to depolarised arrest possibly by reducing depolarisation-induced Na^+ loading. This polarising arrest effect with I_{Na} blockade has since been explored further and was shown to be effective at different temperatures but 36^oC cardioplegia in the Langendorff perfused rat hearts was the optimal temperature(Asano et al. 2003). It was also used with other additives such as adenosine (Dobson and Jones 2004) or magnesium (Yamaguchi et al. 2007) in experimental studies.

Despite the advantages of lidocaine over hyperkalaemia demonstrated in the experimental studies, the use of Na^+-channel blockade has had minimal clinical uptake bearing in mind that lidocaine is already used clinically as a local anaesthetic and an anti-arrhythmic agent at lower doses. One problem is that lidocaine clearance is dependent on hepatic and renal activity (which is reduced for varying periods as a result of low perfusion during cardiopulmonary bypass), so doses of lidocaine sufficient to induce arrest may lead to accumulation of the drug and its metabolites in the body after reperfusion and cause potential arrhythmic and neurological toxicity (Waller 1981). Yamaguchi and colleagues (Yamaguchi et al. 2007) showed that the lidocaine concentration required to effectively arrest an *in vivo* dog heart was 1.4 mM (in combination with elevated (5.5 mM) magnesium). The plasma concentration in the peripheral circulation was found to be 0.08 mM at the time of weaning from bypass; this is much higher than the upper safety margin defined as 0.03-0.04 mM (Foldes et al. 1960). The study by Yamaguchi and co-workers (Yamaguchi et al. 2007) was the first to look at the toxic effects of high doses of Na^+-channel blockers as cardioplegic agents (Fallouh and Chambers 2007)

1.7.1.3 K^+ channel activation

Drugs that open K^+-channels in the resting cell will tend to shift E_m closer to the potassium equilibrium potential (about -90 mV) under steady-state conditions (Cohen et al. 1993), thereby inducing a hyperpolarised state. E_m is thus shifted away from the activation threshold of the fast Na^+ channels and should lead to cardiac arrest by inhibiting phase 0 of the action potential, making K^+-channel openers potential cardioplegic agents. Pinacidil, which targets the ATP-sensitive K^+ (K_{ATP}) channels, was found to be superior to STH as a cardioplegic agent in an *in vivo* pig heart model (Jayawant et al. 1999). More recently, activation of K_{ATP} channels was shown to reduce myocyte Ca^{2+} loading and to reduce post-ischaemic contracture in the isolated myocytes (Baczko et al. 2005). However, the efficacy of pinacidil to induce cardiac arrest has been questioned (Walgama et al. 2000); our group has shown that, even with very high concentrations, pinacidil failed to induce arrest in aerobically-perfused rat, rabbit or guinea pig hearts (with species-dependent concentration effects). Furthermore, the prolonged electrical and mechanical activity with pinacidil cardioplegia in the rabbit hearts (~900 seconds and ~200 seconds, respectively) suggests arrest was ischaemia-induced rather than by direct effects on the K_{ATP} channels (Jayawant and Damiano 1998). Additionally, it was found that, with pinacidil cardioplegia, there is an increase in oxygen consumption post-ischaemia (Lawton et al. 1997) compared with STH group in the rabbit hearts; this was explained by the oxygen demand for high reparative processes of viable myocytes or to a higher oxygen debt generated during ischaemia. The questionable ability of pinacidil to induce arrest as well as the increased oxygen demand at reperfusion presents a significant limitation to pinacidil cardioplegia. More importantly, however, the pinacidil half-life in a healthy human subject was shown to be >2 hours (Ward et al. 1984). This slow clearance, together with a narrow safety margin in the micromolar range (Kowaltowski et al. 2001), means that the high concentrations needed to induce cardioplegic arrest (Lawton et al. 1996) are likely to cause lingering hypotensive and dysrhythmic effects in patients after weaning from cardiopulmonary bypass.

Adenosine, another clinically-useful compound, has been shown to act as a hyperpolarising agent in rabbit SA node cells, with concentrations of ~50 µM inducing

a hyperpolarisation of 12-15 mV and arrest of spontaneous beating in myocytes isolated from the rabbit's SA node (Belardinelli et al. 1988). In bovine and guinea pig isolated ventricular myocytes, adenosine had no direct effect on contraction, resting potentials or action potentials. However, it demonstrated an antagonist effect on the prolongation of the action potential caused by the influx of calcium by isoproterenol and almost inhibited it completely. It also inhibited the positive inotropic effect of isoproterenol (Belardinelli and Isenberg 1983). The binding of adenosine to A_1 purinergic receptors is coupled to the opening of the acetylcholine (Ach)-activated K^+-channel (K_{Ach}), and it can also activate the K_{ATP}-channel with the associated additional cardioprotective effects (Kirsch et al. 1990). Adenosine (0.2-10 mM) has been used to induce arrest in isolated hearts with varying effects on recovery of function; a high dose of (10mM) adenosine alone offered a superior cardioplegic hypothermic arrest (10^oC) over hyperkalaemia only or adenosine + hyperkalaemia combined in the isolated rat heart with improved recovery and conservation of tissue creatine phosphate (Schubert et al. 1989). Meanwhile, Jayawant demonstrated that up to 0.2 mM adenosine in an invivo rabbit heart has slightly reduced functional recovery following normothermic ischaemia compared with pinacidil (Jayawant and Damiano 1998). The main problem with the use of adenosine at this low concentration is, again, the prolonged time to achieve electrical and mechanical arrest of around 20 min and 7 min respectively, which suggests that the mode of arrest was probably ischaemia rather than chemical.

As an additive to hyperkalaemic cardioplegia, adenosine (0.5-2.0 mM) was shown to be beneficial in cardiac surgery patients (Mentzer et al. 1999). Recently, Dobson and coworkers showed that a cardioplegic solution utilising a combination of adenosine (0.2 mM) and lidocaine (0.5 mM) offered significantly improved protection over STH in isolated working rat hearts (Dobson and Jones 2004) and had similar protection to STH in an *in-vivo* dog heart preparation (Corvera et al. 2005). Similarly, adenosine (1.2 mM) combined with procaine (1 mM) also showed superior protection over hyperkalaemia in an in-vivo pig heart model (Jakobsen et al. 2007).

Although the use of adenosine as the sole arresting agent might not be practical due to its extremely short half-life in blood (50-90 sec) (Blardi et al. 1993), it could still provide additive benefit to other arresting agents if added to blood-free solutions.

1.7.2 Inhibiting Ca^{2+} activation of the myofilaments

1.7.2.1 Hypocalcaemia

The Bretschneider cardioplegic solution, a Ca^{2+}-free, Na^{+}-depleted solution developed in the early 1960's, was one of the first solutions to be routinely used clinically (Bretschneider 1964). Later, this solution was slightly modified and Ketoglutarate, Histidine HCl and Tryptophan were added to formulate the Bretschneider-HTK solution (Custodiol®), which is still successfully used today. A Ca^{2+}-free extracellular media prevents Ca^{2+} influx through the L-type Ca^{2+}-channels during the plateau phase or phase 2 of the action potential (Figure 1.4). This inhibits Ca^{2+}-induced Ca^{2+} release from the SR, so abolishing the intracellular Ca_{tr} and preventing activation of the myofilaments (Bers 2002). However, Ca^{2+}-free solutions, although effective at inducing arrest, are limited by the "calcium paradox", in which massive Ca^{2+} overloading of the cell occurs during reperfusion with a Ca^{2+}-containing solution; this is caused by Na^{+}, loaded into the myocyte during the Ca^{2+}-free period, being replaced by Ca^{2+} via the Na^{+}/Ca^{2+} exchanger when the solution with normal extracellular Ca^{2+} levels is reintroduced (Chapman and Tunstall 1987). The calcium paradox is avoided, however, by the reduction of the extracellular Na^{+} concentration. Bretschneider cardioplegia has been shown to be suboptimal during normothermic ischaemia, in contrast to STH, which is protective both in hypothermia and normothermia (Jynge et al. 1978). In addition, the large infusion volumes of Bretschneider solution (which are needed to remove all the extracellular Ca^{2+}) require prolonged infusion times making it unwieldy to use from a clinical perspective.

1.7.2.2 Ca^{2+}-channel blockade

Blocking the calcium channels decreases Ca^{2+} influx and so inhibits Ca^{2+}-induced Ca^{2+}-release from the SR. Theoretically, this is an effective concept to induce arrest in diastole during phase 2 of the action potential (Figure 1.4). Vouhe and colleagues (Vouhe et al. 1980) studied the effect of diltiazem compared with high K^{+} in the *in-vivo* dog's heart and demonstrated better recovery post ischaemia. These myocardial protective characteristics were also tested by the use of calcium channel blockers as an

additive to hyperkalaemic cardioplegia; Yaliniz and colleagues (Yaliniz et al. 2004) demonstrated that small quantities of diltiazem (~1 µM) added to the first and the last infusion of tepid potassium cardioplegia in patients who underwent CABG surgery resulted in slow SA node recovery in the diltiazem group without any major haemodynamic problems. The diltiazem treated group also had less CK-MB rise, which confirms the protective effects of diltiazem. However, the prolonged plasma half-life of current agents (e.g. 2 hours for verapamil (Popovic et al. 2006) and 5.7 hours for diltiazem (Caille et al. 1991)) leads to a long-lasting negative inotropic effect after weaning from cardiopulmonary bypass, which is due to the high affinity of these agents for the L-type Ca^{2+}-channels (Dillon and Nayler 1987). This makes these agents unsuitable to be used as arresting agents due to the slow reversibility.

The $I_{Ca,L}$ channels can also be blocked by elevated extracellular Mg^{2+} concentration (Iseri and French 1984), despite being less effective than hyperkalaemia at inducing arrest (Shattock et al. 1987). Hearse and colleagues (Hearse et al. 1978) demonstrated that the addition of elevated Mg^{2+} (16mM) to hyperkalaemic cardioplegia significantly improved recovery (see 1.7.1.1 above; elevated Mg^{2+} is a basic component of STH cardioplegia. More recently, another study from our lab (Maruyama and Chambers 2008) investigated the value of using high Mg^{2+} as the arresting agent in a novel non-phosphate buffered crystalloid solution (Aqix®RS-I) for a cardioplegic solution. It was studied in Langendorff perfused rat hearts in comparison with STH2 cardioplegia. Concentration-response study demonstrated that optimal Mg^{2+}concentration was 25 mM, the Mg^{2+} arrested hearts also developed a delayed and less profound ischaemic contracture compared with STH2. As for the left ventricular developed pressure (LVDP) recovery, the data suggest that Aqix+Mg^{2+} cardioplegia could offer superior recovery if used frequently (20 minutes) in normothermic ischaemia

Due to the easy reversibility of Mg^{2+}, hypermagnesaemia is probably the most clinically relevant calcium channel blocking agent for use in cardioplegia.

1.7.2.3 Influencing the sensitivity of myofilaments to Ca^{2+}

2,3-butanedione monoxime (BDM), originally developed as an antidote to sarin poisoning, was found to arrest cardiac myocytes without altering the calcium transient. Vahl and colleagues (Vahl et al. 1995) demonstrated in the human ventricular muscle strips loaded with the calcium dye, Fura-2, that 30 mM of BDM completely inhibited the force generated by contraction with only partial reduction in the calcium transient. BDM induces inhibition of force-producing cross-bridge formation within the myofibrils (Gwathmey et al. 1991) despite the rise in intracellular calcium levels during the action potential. Snabitis and colleagues (Snabaitis and Chambers 1999) studied the effect of BDM as an additive to a "polarising" solution using TTX as the arresting agent during long term preservation storage studies (8 hours of ischaemia) in working mode rat heart preparations. They demonstrated that BDM (30 mM) increased aortic flow recovery from 56% to 76% of control when added to a preservation solution constituting the sodium channel blocker TTX, the Na^+/H^+ exchanger inhibitor [HOE694] and the $Na^+/K^+/2Cl^-$ co-transporter inhibitor furosemide. This offered significantly improved preservation compared with STH2 solution with recovery of 26%. Jayawant and colleagues (Jayawant et al. 1999) studied the concept of cardioplegic arrest using BDM as an arresting agent rather than as an additive in rabbit perfused hearts undergoing ischaemia with multiple infusions of blood-based cardioplegia. BDM (20 mM) offered LVDP recovery comparable to STH2 cardioplegia. The BDM group, however, had much shorter atrio-ventricular (A/V) interval with lower chance of complete heart block compared with the control and STH groups.

Like many other arresting agents mentioned earlier, despite the experimental evidence of the advantages offered by BDM, the use of it in myocardial protection was never translated to the clinical arena. A possible explanation for this might be that BDM concentrations required to induce complete arrest or offer any protection are very high (20-30 mM), with a potentially strong calcium chelating effect that may make it too toxic. In summary, *in vitro* evidence suggests an advantage in using BDM as an additive to high K^+ cardioplegia for better myocardial protection in the rat (Snabaitis and Chambers 1999). What is not very clear is how safe are these protective doses of BDM and how wide is the benefit safety margin.

1.8 Alternative methods of myocardial protection during surgery

Surgeons had to operate on the beating heart prior to the development of cardiopulmonary bypass and the first operation performed for coronary artery disease was on a beating heart in 1936 (Marvin 1941). The introduction of cardiopulmonary bypass (CPB), however, ensured cardiac surgery became a great success due to the ease and the predictability of operating on an arrested heart by hyperkalaemic cardioplegia and CPB-supported circulation (Pfister et al. 1992). Interestingly, in 1972, and hardly a decade passed with the comfort of operating on an arrested heart, Ankeney reported his experience of performing CABG on beating hearts without the support of cardiopulmonary bypass to The Society of Thoracic Surgeons in the USA (Pfister et al. 1992). This has started a very wide trend worldwide to perform coronary surgery on beating heart and becomes a well established technique called off pump surgery. Off pump surgery is beyond the scope of our work, which mainly investigates strategies for myocardial protection against global ischaemia during cardiopulmonary bypass. However, the modern experience of off pump surgery along with the culture of exploring alternative strategies to improve outcome resulted in the following alternative methods to conventional cardioplegic arrest.

1.8.1 Intermittent cross-clamp fibrillation

Intermittent cross clamp fibrillation (ICCF) is a well established method of cardioprotection during coronary artery bypass (CABG) surgery, which offers a relatively quiescent and bloodless surgical field during CABG. This technique involves exposing the heart to intermittent short episodes of global ischaemia (around 10 min each) while the surgeon is constructing the distal coronary anastomosis of each graft. The heart is maintained in ventricular fibrillation (VF) during this period, with the heart being fully reperfused and defibrillated at the end of each CFF. In 2004, a UK survey (Karthik et al. 2004) showed that 17% of surgeons continued to use this strategy in coronary surgery as their predominant method of myocardial protection during CABG. The advantage of this technique is to minimise the length of ischaemia to 10 to 15 minutes at a time and establish reperfusion as soon as possible, as it is thought that the myocardium can tolerate this levels of ischaemia without sustaining irreversible injury.

Additionally, intermittent cross-clamping would mimic ischaemic preconditioning by exposing the myocardium to short periods of ischaemia (Abd-Elfattah et al. 1995; Fujii and Chambers 2005) and makes the heart more tolerant to subsequent periods of ischaemia while performing the subsequent grafts. Numerous studies were conducted in order to determine the effectiveness of this method over cardioplegic arrest and showed comparable results despite common belief that cross-clamp fibrillation results in higher cardiac enzyme release and embolic events due to the constant handling of the aorta (Scarci et al. 2009) and the concern of inadequate perfusion between the consecutive episodes of ischaemia (Dunning et al. 2006). There is no evidence relating to the trend for using cross-clamp fibrillation since 2004; however, as patients becoming sicker with more diffused disease being operated on with multiple vascular targets, it is likely that this method for myocardial protection is in decline. We analysed retrospective data from STH, and showed that in patients with EuroSCORE (European System for Cardiac Operative Risk Evaluation) more than 9, ICCF gave better long term survival than cardioplegia (Scarci 2010). This study has its limitation as it looked at the mortality rates retrospectively without taking into account the cause of death in both groups. Therefore the endpoint of this study might not be reflecting the best myocardial protection strategy in these two groups. Equally, a study by Dunning and colleagues demonstrated that the reperfusion between the episodes of cross clamps in this method of protection can be variable and unreliable (Dunning et al. 2006).

1.8.2 Systemic hypothermia and a non-ischaemic elective fibrillatory arrest

This technique is rarely used; it is performed without cross-clamping the aorta or exposing the heart to ischaemia. Systemic hypothermia (28°C) is achieved with cardiopulmonary bypass and elective fibrillatory arrest is induced with maintenance of systemic perfusion pressure at between 80 and 100 mm Hg; allowing certain procedures like redo surgery with patent grafts from previous CABG to be performed safely; In 1984, Akins and colleagues (Akins 1984) reported a low incidence of perioperative infarction and a low hospital mortality rate in 500 consecutive patients using this technique. The limitations of this technique include the bloody surgical field during surgery without cross-clamping the aorta, which therefore limits its use to CABG and sometimes mitral surgery. Less importantly ventricular fibrillation is associated with

increased muscular tone, which can limit the surgeon's ability to position the heart for optimal exposure. It is generally not applicable for intracardiac procedures (Okamura et al. 1996); however, certain modifications can be adopted in order to overcome these limitations and perform intra-cardiac procedures like mitral valve surgery adopted when it is difficult to induce ischaemia in redo surgery with patent coronary grafts. In these situations, cross-clamping can be applied to offer a relatively bloodless field or alternatively, low systemic perfusion pressures with further cooling (Byrne et al. 1999).

1.8.3 Minimal myocardial contraction

This strategy was first explored clinically in the early nineties by Sweeney and Frazier in patients with very poor ventricles by supporting circulation with partial bypass using ventricular assist devices and inhibition of contraction using high doses of the short-acting β-blocker esmolol (Sweeney and Frazier 1992). This ensured avoidance of ischemia by continuous perfusion in this sick group of patients with a relatively flaccid and slow heart by using esmolol, which assists the surgeon to construct good quality anastomosis. The introduction of esmolol by Sweeney and colleagues was through a unique strategy, which offers middle ground between off-pump and on-pump surgery and resulted in a very good outcome to patients with poor prognosis due to their very poor ventricular function. Kuhn-Regnier (Kuhn-Regnier et al. 1999) and colleagues have adopted this concept of reducing contraction using systemic esmolol effusion and applied it to coronary artery surgery with CPB, aortic cross-clamp and direct continuous coronary perfusion with esmolol enriched blood. This method offered the advantage of avoiding ischaemia while operating on an empty heart that was relatively easy to manipulate. It was also used for performing intra-cardiac surgery by Scorcin and colleagues (Scorsin et al. 2003) and who used the term minimal ventricular contraction. The use of esmolol in cardiac surgery was not limited to minimal ventricular contraction; it was used as an adjunct to conventional cardioplegia by us (Fujii et al. 2006) and others (Booth et al. 2002; Boldt et al. 2004). It was also used in off-pump surgery (Arlock et al. 2005) and as an alternative to conventional cardioplegia experimentally in our lab (Bessho and Chambers 2001; Bessho and Chambers 2002) and by others (Geissler et al. 2000).

The short-acting β-blocker, esmolol, was used in various settings in cardiac surgery aside minimal ventricular contraction; the following section will shed some light on the development of β-blockers and explore the benefit of esmolol in cardiac surgery.

1.9 β-blockers and esmolol

The β-receptors are part of the sympathetic nervous system which respond to catecholamine (adrenaline and noradrenaline) (Ahlquist 1948). The β-receptors relevant to the heart are β_1 receptors and they are activated by coupling with G_s protein which increases cAMP resulting in increase contraction tachycardia (*Drake* 2005). The development of the first β-blocker propranolol in 1962 (Black and Stephenson 1962) has revolutionised the treatment of ischaemic heart disease, hypertension and arrhythmia (Harrison et al. 1965; Waal 1966; Mason et al. 1969). However, propranolol was a non-selective β blocker which was responsible for its non cardiac side effect. This has resulted in developing more cardiac selective β-blockers targeting the β_1 cardiac receptors which led the development of a group of β-blockers like atenolol, bisoprolol and metoprolol. These agents remained the cornerstone in the treatment of ischaemic heart disease, arrhythmia and hypertension until today (Cruickshank 2000). More recently agents like carvedilol, which are less selective to β_1 receptors have demonstrated increased benefit in the treatment of heart failure due its additional alpha blocking activity (Packer et al. 1996). Interestingly, recent evidence suggests that the variation of selectivity among different β-blockers is not as profound as it was thought to be (Owens 2005).

In order to extend the benefit of β-blockers beyond its chronic use in angina and chronic hypertension, the development of an ultra-short acting β_1 selective blocker esmolol has led to expanding its use to treating other conditions like acute hypertension in critical care and cardiac surgery as explained earlier. As esmolol is main scope of this work the following section looks at esmolol in details.

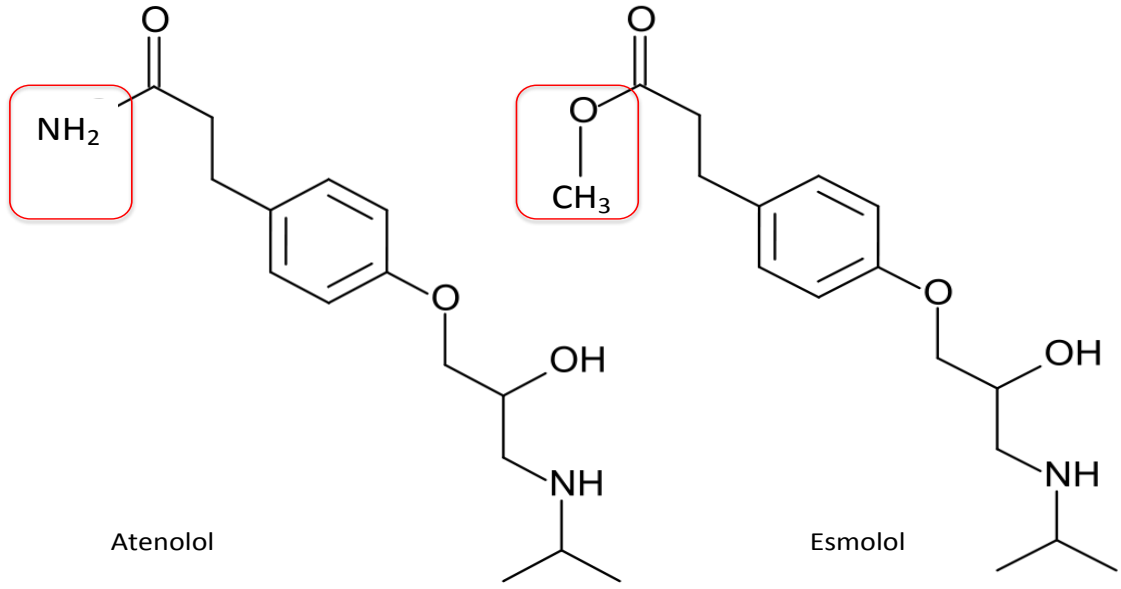


Figure 1.12 Esmolol molecular formula with the ester link (squared) compared with atenolol which is responsible for the fast metabolisation in the red cells

Esmolol (ASL-8052), an ultra-short acting cardioselective beta-blocker, was first developed by Edhart and colleagues (Erhardt et al. 1983) in 1983 and is a phenoxy-propranolamine with a half-life *in-vivo* of 9 minutes. The molecule has an ester link in the para-position of the phenyl ring (Figure 1.12) that is responsible for the esmolol cardioselectivity and ultra-short duration of action of the drug (Zaroslinski et al. 1982). It is registered in the UK for the treatment of supraventricular tachycardia, post-operative hypertension (Gray et al. 1985) and tachycardia syndrome according to the British National Formulary (BNF). Roth and colleagues studied the effect of esmolol on reperfusion injury in regional ischaemia in an *in-vivo* canine heart preparation using LAD artery ligation and demonstrated that the infusion of esmolol at reperfusion decreased free radical mediated injury (Roth and Torok 1991) and inhibited the inflammatory response (Roth et al. 1995). The anti-ischaemic properties of esmolol were examined clinically in patients with unstable angina by Hohnloser and colleagues (Hohnloser et al. 1991); esmolol was administered in patients with diagnosed unstable angina and was titrated against blood pressure (BP) and heart rate. Compared with the placebo group, there was significant reduction in the need to proceed to urgent revascularisation and lower tendency to develop silent angina in the esmolol treated group.

The bradycardic effect of esmolol, as well as the mounting evidence on its protective effects against ischaemia, encouraged many groups to explore its value in cardiac surgery. It was used by Booth and colleagues (Booth et al. 2002) to assess any protective effect of systemic infusion of esmolol during CPB and cold hyperkalaemic cardioplegia in a canine preparation with prior LAD occlusion on post ischaemic function, to mimic CABG patients. The β-adrenergic receptor density in biopsies taken from the ventricles was comparable in both the esmolol and placebo groups; however, isoproterenol-stimulated adenylyl cyclase activity was significantly improved post-bypass in the esmolol group. This can explain the improved systolic contraction post-CPB, possibly by better inotropic response to catecholamine. Clinically, Boldt and colleagues (Boldt et al. 2004) studied esmolol in combination with the phosphodiesterase III inhibitor, enoximone, as a supportive drug for its myocardial protective specifications against global ischaemia. The combination was administered in the perioperative period as a systemic infusion in patients over the age of 65 undergoing CABG. Compared with placebo, the treatment group had a better myocardial protection profile, and presented with higher cardiac index and less troponin and CK rise post operatively. There was also less rising in the plasma inflammatory markers interleukins 6 and 8 levels and in polymorphonuclear elastase levels in the treatment group. Meanwhile work from our lab (Fujii et al. 2006) examined the use of esmolol as an additive to blood potassium cardioplegia in the isolated rat hearts and showed no added protection.

In a different application, esmolol was used as an alternative to cardioplegia rather than as an adjunctive measure of myocardial protection. The concept of minimal ventricular activity by esmolol explained earlier was examined experimentally by Geissler and colleagues (Geissler et al. 2000). They used an *in-vivo* canine cardiopulmonary bypass (CPB) similar to the model described earlier by Booth and colleagues (Booth et al. 2002) to mimic emergency CABG. After establishing CPB, the dogs were randomised into two groups; one received warm blood cardioplegia and the other warm blood with high doses of esmolol in the aortic root. Myocardial water content as well as global and regional LV function were measured post-CPB. There was comparable LV function in both groups; meanwhile, the esmolol group demonstrated lower water contents and less oedema. Clinically, the same concept of minimal ventricular activity by esmolol was

compared with high potassium blood cardioplegic arrest by Kuhn-Regnier and colleagues (Kuhn-Regnier et al. 1999) in a randomised controlled trial of 60 low-risk patients undergoing CABG. There was comparable outcome in both groups in terms of post-operative complications, inotropic support, enzyme release and ICU length of stay. However, there was significant improvements in cardiac index measurements in the esmolol group 4 hours post-operatively. Similarly, in the esmolol group there was a significantly decreased lactate release in the coronary sinus during CPB. The same group reported their experience in using esmolol in the same manner in 200 high risk patients with average EuroSCORE of 6 undergoing CABG operation (Kuhn-Regnier et al. 2002). They demonstrated morbidity and a mortality rates within the expected range as per EuroSCORE. The authors then concluded that esmolol continuous warm infusion into the coronary arteries is a safe and effective technique in CABG procedures; it may be especially advantageous in high-risk patients.

The use of esmolol as an alternative to cardioplegia explained thus far has been to maintain continuous perfusion minimal ventricular contraction with no ischaemia or arrest. This was shown to be a useful mean of performing cardiac surgery however it does not offer the advantage of a bloodless and quiescent surgical field offered by conventional cardioplegia (see Section 1.6.1). Work from our lab (Bessho and Chambers 2001) has shown that high concentrations of esmolol (~1.0 mM), can induce cardiac arrest in the Langendorff perfused hearts. Esmolol was, therefore, used as a conventional arresting cardioplegic agent in these experiments rather than minimal ventricular activity. The optimal method of esmolol cardioplegia was studied comparing intermittent versus continuous administration and flow controlled versus pressure controlled coronary perfusion. It was concluded that the use of high doses of esmolol was proven to be a safe cardioplegic agent and, when given in an oxygenated perfusate, was superior to St. Thomas' Hospital cardioplegia or cross-clamp fibrillation in terms of LVDP recovery after prolonged periods of ischaemia (Bessho and Chambers 2001; Bessho and Chambers 2002). However, the mechanism of cardiac arrest induced by esmolol in these studies is not clear and could not be entirely explained by its β-blocking effect, especially in a Langendorff heart preparation the existence of a catecholamine background release is not very clear; Adrenaline for instance is catecholamine with β receptor agonism properties and therefore will be responsible for

any sympathetic effect on the heart. Potentially, it can be released from the nerve endings which would have been divided during harvesting the heart. These sympathetic nerve endings are formed by the axons of the postganglionic sympathetic neurones placed in the thoracolumbar sympathetic chain of the spine (*Drake* 2005), any possible adrenaline activity in this model is not likely to be prominent after the stabilisation period of the experiment. In these experiments esmolol had a negative inotropic effect at high concentrations, which resulted in 25% decrease in LVDP at 0.1 mM without significant effect on heart rate. This negative inotropic effect progressed to a complete ventricular arrest at 1 mM with ongoing atrial activity (Figure 1.13). These findings suggest that esmolol-induced arrest could be caused by the failure of contraction due to the profound negative inotropic effect. It is also possible that dissociation between atrial and ventricular activity at 1 mM could be due to either complete heart block at the level of the AV node or the downstream conduction system, which again can explain the arrest by esmolol (at 1 mM). Alternatively, this might be simply due to poor atrial perfusion in this preparation, which reduced esmolol bioavailability so it failed to inhibit contraction in the atria. All these possible mechanisms can explain the arrest by esmolol (1 mM) but none suggests any involvement of the β-receptors in hearts isolated from any catecholamine background. One possible effect of esmolol in such preparation on β-receptors is by acting as an inverse-agonist and activating PKA-dependent mechanisms (Baker et al. 2003). However, this effect would be expected to induce positive inotropic effect and to not require such high concentrations. Therefore, there must be another mechanism by, which high dose esmolol is able to induce cardiac arrest independent from its β-blocking effect.

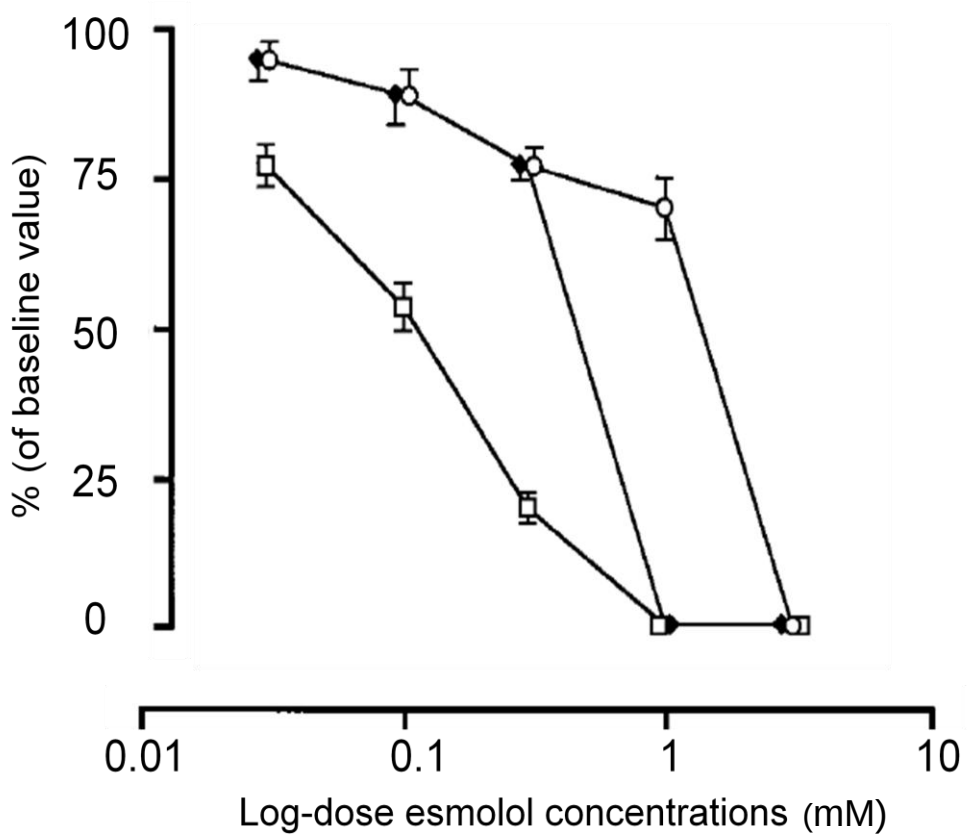
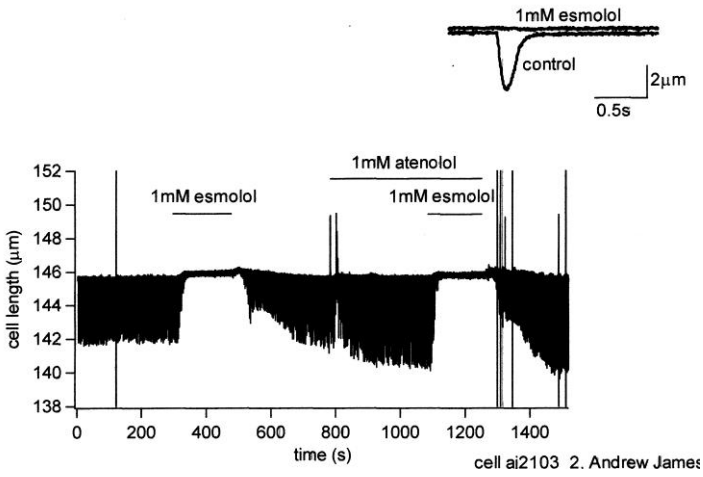


Figure 1.13 Log dose response curves of LVDP (open squares), ventricular rate (closed diamonds), and atrial rate (open circles), with increasing concentrations of esmolol in KHB as percentage of the baseline value. (from Bessho and colleagues (Bessho and Chambers 2001))

In order to establish the mechanism of esmolol arrest or even its negative ventricular inotropic effect in the isolated heart, we conducted some pilot studies in isolated rat cardiac myocytes. The isolated rat ventricular myocyte loaded with the Ca^{2+} sensitive dye, Indo-1 and were electrically-stimulated. The myocyte contraction was studied in an edge-detection and the Ca^{2+} transient was measured using fluorescence microscopy (Figure 1.14). Esmolol (at 1 mM) inhibited myocyte shortening completely (i.e. induced arrest); however, atenolol (at 1 mM) had no effect on myocyte shortening. Infusion of esmolol (1mM) on an atenolol (when the β-receptors should be blocked) still induced complete inhibition of myocyte shortening. Despite arresting the myocyte, esmolol did not abolish the Ca^{2+} transient although there was a decline of around 40%, suggesting that esmolol could have a desensitising effect on the myofilaments to calcium. Additionally, this suppressive effect on the Ca^{2+} transient could be either by blocking

the Ca^{2+} entry into the cell via the voltage dependent L-type Ca^{2+} channels or by blocking the Ca^{2+}-induced Ca^{2+} release from the SR.

A



B

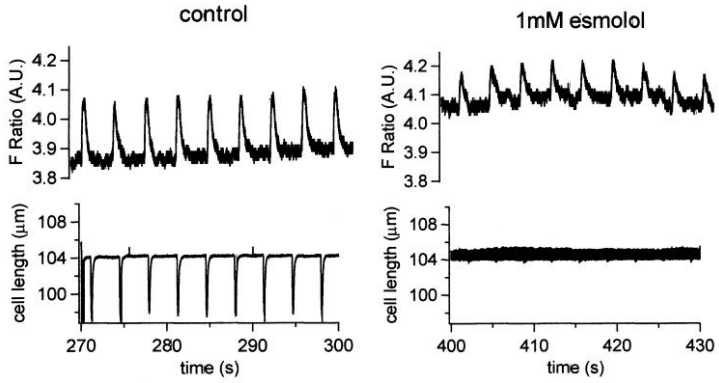


Figure 1.14 Isolated rat ventricular myocyte electrically stimulated with cell length measured using cell edge detection and Ca transient measured using Indo-1 (pilot data performed by A James and colleagues)

1.10 **Hypothesis and Aims**

These different mechanisms mentioned above, which can explain the arresting mode of action of esmolol suggest the following hypotheses:

1. Esmolol has a negative inotropic effect and induces cardiac arrest through mechanism(s) independent from its β-adrenoceptor blocking effect.
2. The decreased Ca^{2+} transient (panel B, Figure 1.14) suggests that esmolol might influence the L-type calcium channels or act directly on the SR at intracellular level.
3. The abolition of myocyte shortening and the reduced calcium transient (panel B of Figure 1.14) suggests a possible direct desensitising effect of esmolol on the myofilaments to calcium.
4. The atrio-ventricular dissociation induced by esmolol at (Figure 1.13) suggests possible effects on the conduction system of the heart.
5. The use of esmolol as an arresting agent could offer a new mechanism for induction of cardioplegic arrest that is superior to hyperkalaemic arrest.

The aims of our studies to address these hypotheses are:

1. To study the effect of esmolol on contraction and the calcium transient in isolated myocytes.
2. To study the effect of esmolol on the myofilaments sensitivity to calcium in isolated skinned myocytes.
3. To study the effect of esmolol on the L-type calcium channel and the sarcoplasmic reticulum (SR).
4. To study the arresting effect of esmolol on the conduction system in Langendorff perfused hearts.
5. To investigate the effects of esmolol as a cardioplegic agent on whole heart preparation as a basis for developing an alternative cardioplegic solution to hyperkalaemia.

1.11 Key Characteristics of the difference between the rat heart and the human heart

The rat heart has always been a good way to conduct cardiac research and major discoveries have been made from working on the rat heart like the use of ACEI following MI (Pfeffer et al. 1985; Pfeffer et al. 1985), the use of endothelin receptor agonist in the treatment of pulmonary hypertension (Sakai et al. 1996) and many more examples. However, there are important anatomical and physiological differences between the rat hearts and the human hearts beyond the difference in size, which should be considered during any experimental work and are explained here:

1.11.1 Anatomical variations

The issue with size difference is very important issue in our studies as the small coronary arteries of the rat hearts could potentially make the application of any coronary treatment dependent on the viscosity of this preparation in the rat more than that in the human. This was noted in studies we conducted using esmolol and blood cardioplegia which resulted in slower coronary perfusion in the rat heart compared to crystalloid preparations (Fujii et al. 2006).

Other anatomical variations are also important; for instance the coronary artery do not lie on the surface of the myocardium like the human hearts, instead they have an intra-myocardial course (Halpern 1957). The septum is usually largly supplied by the right coronary artery with the largest septal branches arising from the origin of the RCA. The circumflex artery is ill defined in the rats as well. More importantly the blood supply to the heart is not limited by the coronary artery but also gets some supply from extra-cardiac branches like the cardiacmediastinal arteries which gives rises to the internal mammary arteries (Halpern 1957).

Another important variation is the presence of a left and right superior vena cava (SVC) instead of an innominate vein in the rats. The right SVC drains in the usual site on the

right atrium while the left drains into the coronary sinus. We have been fully mindful of these variations in designing out experimental protocols

1.11.2 Electrophysiological variations

The SA node in the rat is situated at the junction of the right SVC and the right atrium. However, the blood supply to the SA nodes originates from the extra cardiac branches explained above (Halpern 1957). The heart rate of the rat is significantly faster than human heart which makes the assessing the ECG for ischaemic changes difficult. Otherwise, morphological studies examining the intracardiac nerve plexus in the rat hearts using acetylcholenesterase histochemistry methodology demonstrated that the intrinsic nerve supply fits within the same anatomical scheme as the human nerve plexus. This suggests that the rat model offers a suitable alternative to investigate the effect of the intrinsic nerve supply on the heart (Batulevicius et al. 2004). This is particularly relevant to investigate cardioplegic arrest in the heart. In summary, it seems that the mammal's hearts in general has a common micro and macro anatomical structure in the conduction apparatus and any adaptation was achieved in the function of the heart (Meijler 1985)

CHAPTER 2. METHODOLOGY

2.1 Animals

In the studies described in this thesis, adult males Wistar rats weighing 240 and 325 g were used. All animals received humane care in accordance with the "Guidance on the Operation of the Animals (Scientific Procedures) Act of 1986" (HMSO, London, England)

2.2 Solutions and reagents

Reagents and enzymes were supplied by Sigma-Aldrich Chemicals Ltd. (MO), unless otherwise stated. All solutions were made with ultra-pure water (resistance >16 MΩ).

2.2.1 Tyrode solution for intact isolated myocytes studies

This was prepared by adding the following ingredient (in grams) to 900 ml ultrapure water; NaCl, 8.19; KCl, 0.40; Na_2HPO_4, 0.14; $MgSO_4.7H_2O$, 0.25; HEPES, 1.19; Glucose, 1.8; then 1 ml of 1M concentration stock solution of $CaCl_2$ was added to the mixture. More pure water was added to make the total solution volume to a litre after ensuring all the salts are dissolved. The final concentrations in the solution are (mM): NaCl, 140; KCl, 5.4; Na_2HPO_4, 1; $MgSO_4.7H_2O$, 1; HEPES, 5; glucose, 10 and $CaCl_2$, 1. Then pH was adjusted to 7.45 at the temperature of the experiment with 1M stock NaOH. For the calcium transient measurement, the fluorescent calcium indicator fura-2 AM (Calbiochem) was used as a 2 mM stock solution dissolved in dimethylsulphoxide (DMSO). For the SR blocking studies, the cells were treated with thapsigargin (2 μM) for 20 minutes in a Tyrode solution containing 7 mM $CaCl_2$ instead of 1 mM (for further details see Chapter 5).

2.2.2 Solutions for skinned myocytes

For the skinned myocytes experiments, the solutions were prepared and kept in plastic containers to prevent any contamination with calcium from glassware. Furthermore,

stock solutions of 500 mM N-bis[2 hydroxyethyl]-2-aminoethanesulfonic acid (BES) and 1M Potassium propionate (KPr) were prepared, pH neutralised with 1M KOH, and passed through a chelex-100 column (Bio-rad, CA). Chelex was supplied in the Na^+ form. The chelex beads were changed, initially to the H^+ form by washing in 1 M HCl, and then to the K^+ form using previously-chelexed KOH. During treatment of the solution with chelex, the contaminant Ca^{2+} was removed and K^+ was released into the solutions. The following solutions were then prepared according to the following concentrations:

Ca^{2+}-free relaxing solution (Kentish 1986) contained (in mM): KPr, 70; BES, 100; disodium creatine phosphate (Na_2CP), 10; Na_2H_2ATP, 6.3; $Mg^{2+}ATP$, 5; K_2EGTA, 10; The purity of EGTA was assumed to be 96.3% (Kentish 1986). Na_2H_2ATP and Na_2CP were added as solids. The pH was adjusted to 7.1 at 23°C with 1M chelexed KOH and ionic strength was 200 mM. DTT (1 mM) was added on the day of experiments. To prevent degradation of protein, protease inhibitors (Complete Mini protease inhibitor cocktail tablets) were added to relaxing solution (1 tablet per 10 ml) according to the manufacturer's instructions (Roche Diagnostics, I.N). These inhibit serine proteases, cysteine proteases and metalloproteases. The skinning solution was relaxing solution with 1% Triton X-100 v/v. Storage solution was a mixture of relaxing solution and glycerol (50:50 v/v).

Maximal activating solution was prepared as per relaxing solution but with replacement of K_2EGTA with 10 mM CaK_2EGTA. A CaK_2EGTA stock solution (50 mM) was prepared by heating solid $CaCO_3$ and solid EGTA to 80°C, which combined together to produce CaEGTA as CO_2 was released. The solution was neutralised with Ca^{2+}-free KOH. Various Ca^{2+}-activating solutions were prepared by appropriate combinations of 50 mM K_2EGTA and 50 mM CaK_2EGTA to give a full range of total Ca^{2+} concentrations from zero to 50 mM, corresponding to a range of 0–10 mM in the final solutions. The range of Ca^{2+}-activating solutions, together with the final Ca^{2+} concentrations and calculated pCa values, are shown in Table 2.1.

As a result of the temperature-dependent pKa of BES, at lower temperatures solutions become more alkaline, with a 0.016 pH unit change for each 1°C decline in temperature. Therefore, for experiments conducted at 15°C, the pH was set to 6.94 at RT (23°C)

Total $[Ca^{2+}]$in bathing solution (mM)	*Free $[Ca^{2+}](\mu M)$*	*pCa*
5.5	0.34	6.47
6	0.42	6.38
6.5	0.51	6.29
7	0.65	6.19
7.5	0.83	6.08
8	1.10	5.96
8.5	1.55	5.81
9	2.45	5.61
9.5	5.13	5.29
10	31.62	4.5

Table 2.1 Ca^{2+} solution calculations for the skinned myocyte activating solution

2.2.3 Solutions for $I_{Ca,L}$ patch clamping

Pipette solutions were prepared by adding the following ingredients in 90 mls of ultra-pure water (mM): L-aspartic acid, 1.13; CsOH, 1.43; TEA Cl (tetraethylammonium chloride), 0.33; HEPES, 0.24; EGTA, 0.038; MgATP, 0.2; Na-GTP, 0.005; NaCP,0.16. $MgCl_2$ was prepared as 1 M stock and 0.1 ml. was added. Then the pH was adjusted to 7.2 using CsOH. Ultra pure water was then added to make the solution's volume to 100 mls. The solution was filtered using 0.5 micron cellulose fibre filters. The final

concentrations were; L-aspartic acid, 85; CsOH, 85; TEA Cl (tetraethylammonium chloride), 20; HEPES, 10; EGTA, 1; MgCL2, 1; MgATP, 4; Na-GTP, 0.1; NaCP,5.

The external solution was the Tyrode solution mentioned above for the isolated myocyte studies (Section 2.2.1)

2.2.4 Solution for I_{Na} patch clamping

The pipette solution was prepared as mentioned above in the $I_{Ca,L}$ patch clamping solutions. The final solution contained (mM): NaCl, 5; CsCl, 20; Cs-methanesulfonate, 110; MgCl2, 1; Mg2ATP, 5; HEPES, 5; EGTA, 5; pH 7.2 with CsOH

External solution (mM): NaCl, 5; CsCl, 135; $MgCl_2$, 1; glucose 10; $CaCl_2$, 0.5; $CoCl_2$, 0.5; HEPES, 5; pH 7.4 with CsOH.

A summary of the internal and the external solutions contents is presented later on in Figure 2.8.

2.2.5 Esmolol (Brevibloc® , Baxter)

Esmolol (Brevibloc®) was stored as the concentrated preparation for intravenous infusion at a concentration of 250 mg/ml (755 mM), and was added at the desired concentrations to the Tyrode solution in isolated myocyte experiments, to the external solution in patch clamping experiments and to KHB in the Langendorff perfused heart studies**.**

2.2.6 Atenolol

Atenolol (Sigma Chemicals) was diluted in the Tyrode solution mentioned above at 1 mM concentration.

2.2.7 Solutions for the isolated Langendorff perfused rat hearts

Krebs Henseleit Buffer (KHB) solution was used in the Langendorff perfused rat hearts experiments and contained (mM: NaCl, 118.5; $NaHCO_3$, 25.0; KCl, 4.75; $MgSO_4$, 1.2;

$CaCl_2$, 1.4; and glucose, 11.0), filtered (5 μm pore size) and gassed (95% O2/5% CO2, giving a pH of 7.4 at 37°C).

2.2.8 St. Thomas' Hospital Solution No.2 (STH)

STH was used in the Langendorff perfused rat hearts experiments and contained (mM: NaCl, 110; $NaHCO_3$, 10; KCl, 16; $MgCl_2$, 16 and $CaCl_2$, 1.2), filtered (5 μm pore size).

2.2.9 Lidocaine

Lidocaine (by Nova, UK) local anaesthetic (2%) solution was used as stock solution and was added to KHB solution at the desired concentrations in the Langendorff perfused rat heart studies.

2.2.10 Adenosine

Adenosine (Sigma Chemicals) was diluted in the KHB solution mentioned above at the desired concentrations in the Langendorff perfused rat heart studies.

2.3 Studies using isolated myocytes

2.3.1 Isolation of ventricular cardiomyocytes

Isolation of the ventricular myocytes is a technically complex process. We were fortunate to have the services of a technician trained in this technique. Adult male Wistar rats, weighing 200-250 g were used. Rats were anaesthetised with pentobarbital sodium (50 mg/kg) intra-peritoneally. Then, 200 IU of heparin was injected into the femoral vein. The chest was then opened through a top-roof incision in the abdomen and the heart was exposed and excised. The heart was rapidly mounted on a cannula and perfused through the aorta via a Langendorff perfusion system at a controlled pressure of 100-110 cm of water. The heart was perfused with HEPES buffered Tyrode solution with the following composition (in mM): NaCl, 110; KCl, 5.4; $MgCl_2$, 1.4; NaH_2PO_4, 0.4: HEPES, 4.2; glucose, 10; taurine, 20; creatine, 10 and $CaCl_2$, 1. The pH was adjusted with NaOH at 1 M concentration to 7.35 and the solution was perfused at 37^oC

and gassed with 100% O_2. After 2 minutes of stabilisation, the heart was perfused with calcium-free Tyrode solution (as above but with no $CaCl_2$ and with 0.1 mM EGTA) for 4 minutes, and subsequently with the Tyrode solution and collagenase type II (Worthington Biomedical Corporation) at a concentration of 250 U/ml for 10 minutes. The heart was then removed from the Langendorff perfusion system and the ventricles were isolated and finely minced using sharp scissors. The divided and minced ventricles were then incubated in collagenase solution with bovine serum albumin 1mg/ml for a further 5 minutes. The temperature was kept at 37^{o}C and the solution was always gassed with 100% O_2. The solution was filtered (at 200 µm) into a test tube. The undigested homogenate was incubated for another 5 minutes with the collagenase solution and filtered as before for another two cycles. The myocytes from each cycle were kept separately. Subsequently, they were washed and resuspended in a 1 mM $CaCl_2$ solution. The cells from each cycle were assessed by the shape (rod shaped), the highest yield and the vitality and the best batch was used. The second cycle tended to be the most commonly used. Due to the small number of cells required for the experiments in this project, the myocyte suspension was usually shared with other colleagues in the department to limit the cost and the number of animals used.

2.3.2 Myocyte permeabilisation

Isolated rat ventricular myocytes were permeabilised for the skinned myocyte experiments by re-suspension in the skinning solution (relaxing solution with 1% Triton X-100 v/v) for 15 minutes. Cells were kept on ice during the skinning procedure. The cell suspension was then centrifuged for 3 minutes at 350 g, the supernatant removed and the myocyte pellet resuspended in relaxing solution at 4^{o}C. This cycle of centrifugation and resuspension was repeated three times with relaxing solution to remove any traces of Triton X-100. The myocyte fragments were then resuspended in relaxing solution and used for the calcium sensitivity studies (these experiments were performed with the help of Dr. S. Bardswell).

2.3.3 Apparatus for sarcomere measurement

The apparatus was located in an air-conditioned room to maintain room temperature around 23^{o}C. It consisted of an inverted microscope (Nikon) on an air-balanced anti-

vibration table. The myocyte solution was loaded into a perspex bath with a glass coverslip creating a water-tight base using grease. The bath contained two platinum electrodes along the chamber to stimulate the cells using an IonOptix stimulator with voltage and frequency adjustment capability. The bath was perfused using 8 perfusion channels elevated above the bath and connected to a manifold just before the bath to allow fast switching between solutions. The perfusion lines were heated through temperature-adjusted water jacketing closer to the bath end to eliminate any heat loss. The chamber was mounted above a dry non-phase fluorescence x40 objective lens (Nikon numerical aperture 0.8) of the inverted microscope. The light filters were used in the fluorescence experiment as shown in Figure 2.1.

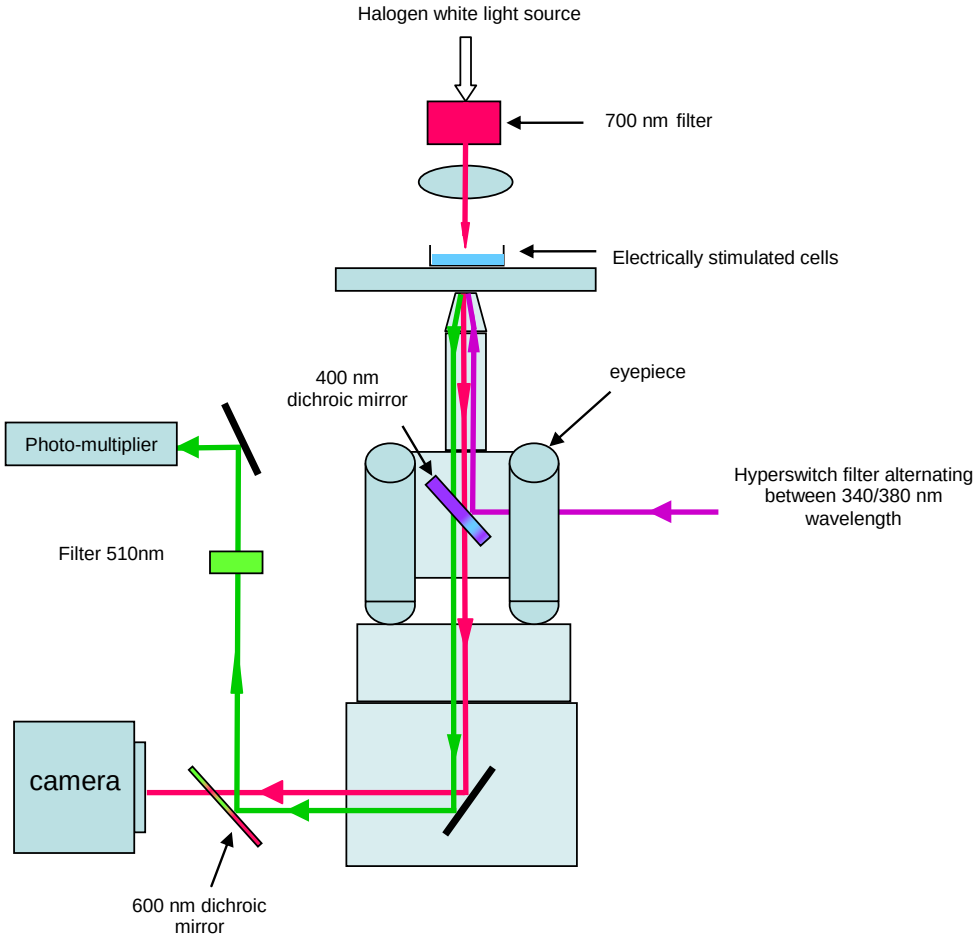


Figure 2.1 Diagram of apparatus for myocyte sarcomere length and calcium transient measurements (modified from Prof. J. Kentish).

The sarcomere length (SL) measurements were studied via a variable field rate video camera connected to the computer and displayed on a separate monitor at a rate of 240 Hz (Figure 2.1). SL measurements were analysed via IonOptix software (Milton, MA, USA). The sarcomere (Figure 2.2) is the contractile unit in the myocyte and it is bordered by the two Z-lines to, which the thin filaments are attached. With the thick myofilaments in the middle it creates alternating dark (A) and light (I) bands, which give the striation appearance. The SL is the distance between the two Z-bands to, which the filaments are attached, with the thick filament in the middle. The IonOptix system uses this striation appearance to calculate the average sarcomere length (SL). IonOptix also registers an event signal on the chart every time there is electrical stimulation in the bath.

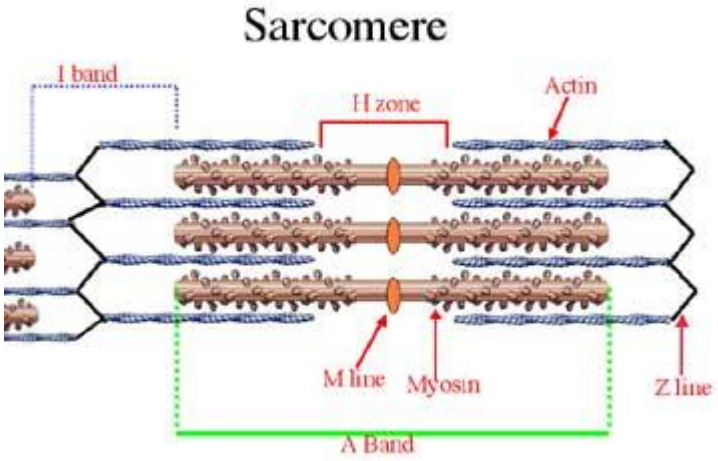


Figure 2.2: Diagram of Sarcomere structure

The sarcomeres are aligned horizontally on the screen by rotating the camera so the striations are aligned vertically. A region of interest (ROI) is then defined (by the operator) in the myocyte by positioning the rectangle with dimensions of about 10x10 µm, which will include around 50 sarcomeres. The larger the ROI, and the more sarcomeres it contains, the more accurate are the results. The pattern of contractions results in the edges of the myocytes to be drawn to the steady centre; this could lead to over-estimating of the sarcomeres away from the centre. Therefore, it is essential to select an area near the centre to ensure that only the sarcomere shortening element is measured and not the drawing effect of the sarcomere movement. The mean SL in the

ROI was calculated using IonOptix software by measuring the sinusoidal light intensity of the dark and light bands in the striations across the video scan lines, i.e, sinusoidal waves with the period equal to the SL (Figure 2.3). Fast Fourier Transform (FFT) analysis calculates the average frequency of the sinusoid. The software calculates the average of the FFTs from each video line; alternatively, it obtains fast measurements of the sarcomere length by measuring single FFT from the average of all scan lines in the ROI. The latter method was chosen as it gives better real time measurements. The statistical power of the FFT measurement is calculated by the software and presented as a peak curve (Figure 2.3); narrow peak indicates more accurate SL measurement with less deviation from the average. This is to exclude poor SL measurements. The difference between the SL at maximum shortening (systole) and maximum resting relaxation (diastole) was calculated and called the sarcomere shortening (SS) as an indication of myocyte contractility in response to electrical stimulation (pacing). The system was calibrated with the help of Prof. JC Kentish, using a novel technique that uses a single diffraction grating of 8.0 µm spacing to check the linearity of the system over a range of sarcomere lengths. The grating was visualised using the x40 objective and the FTT of the striation of the grating was demonstrated on the computer screen as sinusoidal waves evenly spaced. We used 6 peaks to calibrate the system.

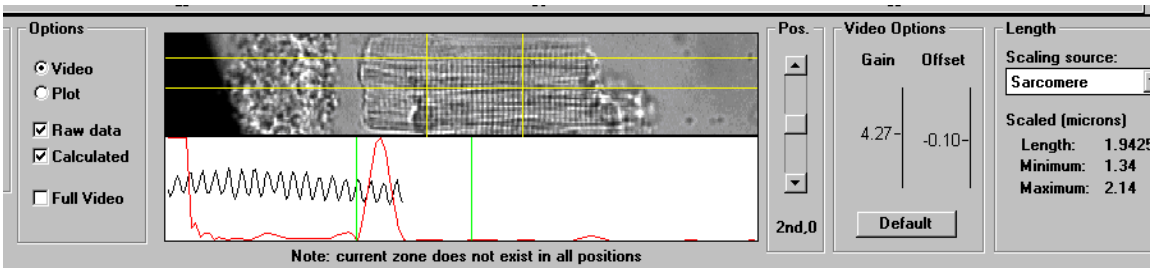


Figure 2.3: Isolated myocyte displayed on IonOptix screen; FTT power line (red)

2.3.4 Apparatus for intracellular calcium measurement

Intracellular calcium was measured using the IonOptix system (Figure 2.1) with the SL measurements in the intact myocyte experiments. The light from the Xenon arc lamp was passed through a hyperswitch filter so the myocytes loaded with the relevant

fluorescence detector (fura-2 AM (Calbiochem) in our experiments) were excited alternately at two wavelengths (340 and 380 nm). The excitation light was reflected onto the myocyte in the bath using a dichroic mirror via the objective x40 lens. The fura-2 fluorescence was then collected and passed through a 510 nm filter onto the photomultiplier to be detected. The fura-2 fluorescence was passed from the photomultiplier to a Cairn Spectrophotometer (Cairn Research, Kent, UK). The spectrophotometer averaged the 340 and the 380 filtered signals and the 340/380 ratio was then calculated and recorded using IonOpix (Figure 2.4). The Ca transient amplitude (Ca_{tr}) was calculated by subtracting the average calcium measurement at the end of each relaxation from its level during contraction.

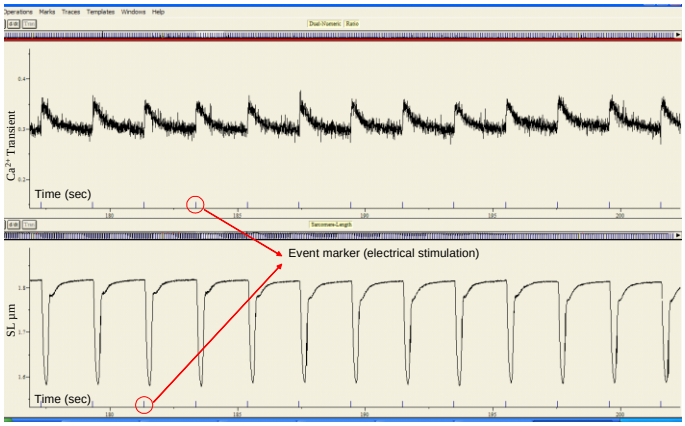


Figure 2.4: Ionoptix recording of the SL and the Ca_{tr} with the event marker indicated

2.3.5 Protocol for the intact cell experiments

Myocytes were incubated with fura-2 at a concentration of 2 µM in Tyrode solution for 10 minutes and then the fura-2 solution was removed and the cells were resuspended in the Tyrode solution and loaded in the system bath (Figure 2.1). After washing the lines and loading them with desired solutions, the bath is filled with the Tyrode solution. The fura-treated cells were then loaded in the bath and electrically stimulated at 1 Hz (unless stated otherwise). SL and Ca_{tr} were measured using the IonOptix system.

2.3.6 Skinned myocyte perfusion system

The cell perfusion set-up was adapted from the method described by Lim and colleagues. (Lim et al. 2001) and a schematic of the system is shown in Figure 2.5. The SF-77B Perfusion Fast-Step system was supplied by Warner Instruments (CT, USA). A single skinned myocyte was gravity-superfused using a three-barrel square glass pipette. A stepper motor was attached to the three-barrel pipette, which served to rapidly switch between adjacent barrels (Figure 2.5 B). The rapid response and low hysteresis of the stepper mechanism allowed for very fast switching times. The three-barrel pipette and stepper motor were attached to micromanipulators to allow precise placement of the pipette tip over the myocyte (Model MN; Narishige Co. Ltd., Tokyo). Up to six different solutions were connected to a single input manifold (which in our experiments were maximal activating solutions with increasing calcium concentration). The contraction generated by increasing the superfused calcium concentrations was measured using IonOptix SL measurements as described. The flow through the barrels was kept constant at ~250 µl/min by adjusting the height of the manifold system. This was measured by recording the decrease in volume of solution in the reservoirs over a 1 min period. This rate of flow prevented mixing of solutions from adjacent barrels, which allowed a single myocyte to be perfused by one solution

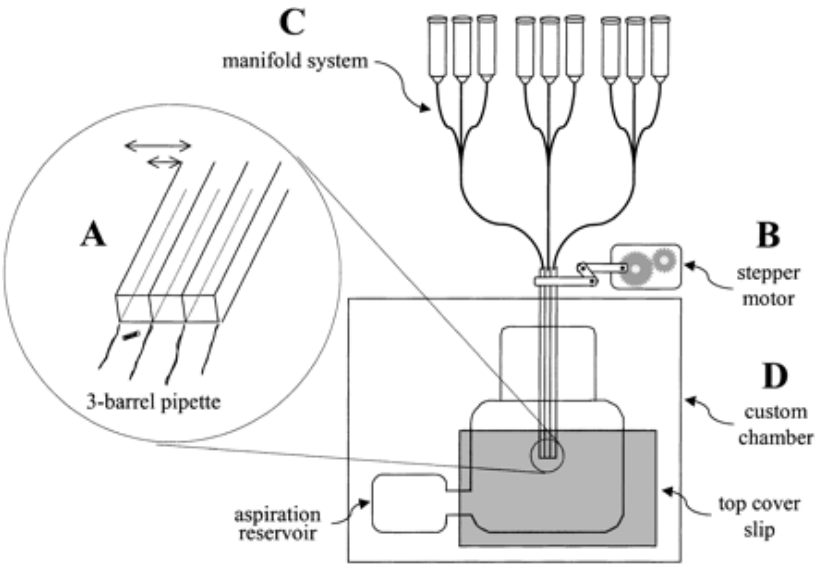


Figure 2.5: Schematic diagram of the perfusion system. A. A 3-barrel pipette was used for perfusion. B. A stepper motor was used to rapidly switch between solutions bathing the myocyte. C. A tap-controlled manifold system was attached to the stepper motor. D. Myocytes were perfused in a custom-made bath. Reproduced from Lim *et al.* (2001) (Lim et al. 2001)

2.3.7 Skinned myocytes experimental protocols

The skinned myocytes were superfused with alternating relaxing and activating solutions at a rising calcium concentration using the stepper-motor. The SL during contraction (Figure 2.6) caused by the rise in calcium in the activating solution was measured with IonOptix and the SL relationship with pCa was analysed.

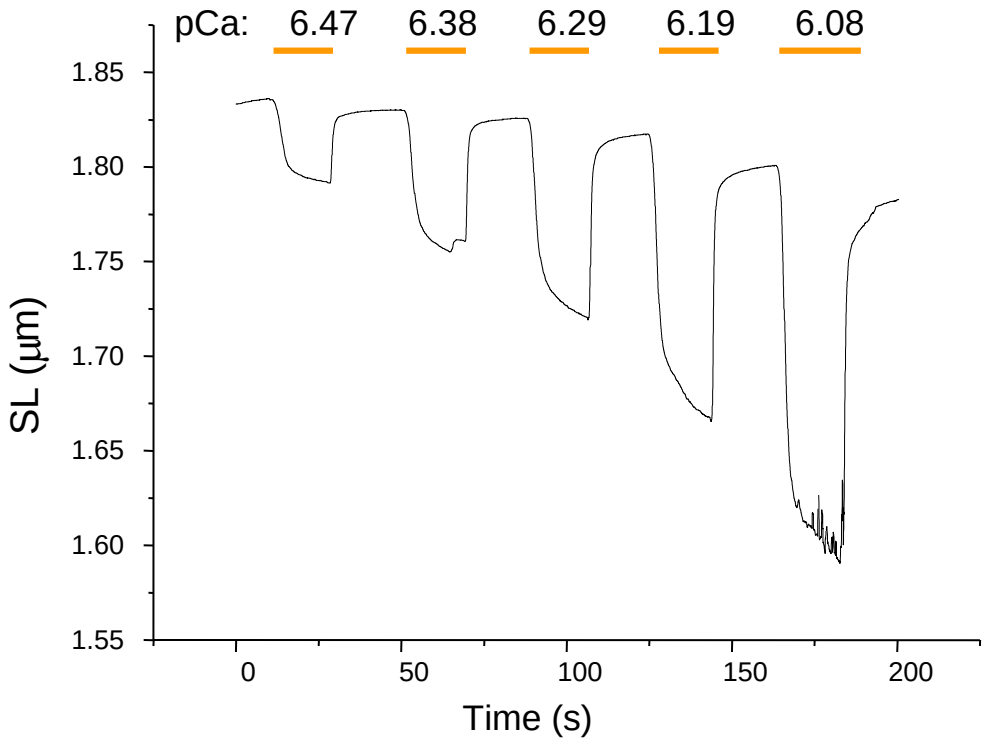


Figure 2.6: Example of skinned myocyte SL shortening induced by rising concentration of calcium (experiment recorded using IonOptix)

2.4 Patch clamping experiments

2.4.1 System set up

Rat ventricular myocytes (isolated as described earlier) were placed in a 2 ml bath on the stage of an inverted microscope (Nikon) with a x40 objective, and also viewed through a Nikon video camera connected to a monitor with larger magnification. The electrode pipettes were attached to a micromanipulator positioned above the bath. The fine movement of the micromanipulator as well as the higher magnification of the video camera helped in applying the pipette to the surface of the myocyte with accuracy. Patch pipettes were pulled from capillary glass with filament (GC150TF-10, Harvard apparatus Co., Novato, CA) using DMZ-Universal Puller (Zeitz pullers, Augsburg, Germany). Pipettes exhibited 1.5 to 2 MΩ resistance when filled with the pipette solution. Currents were filtered at 1 kHz and sampled at 5 kHz. Current acquisition was performed using pCLAMP10® and analysed using CLAMPFIT® (Axon Instruments, Foster City, CA). All recordings were initiated only after 2 minutes to allow complete dialysis of the cytoplasm with a seal resistance not less than 250 MΩ.

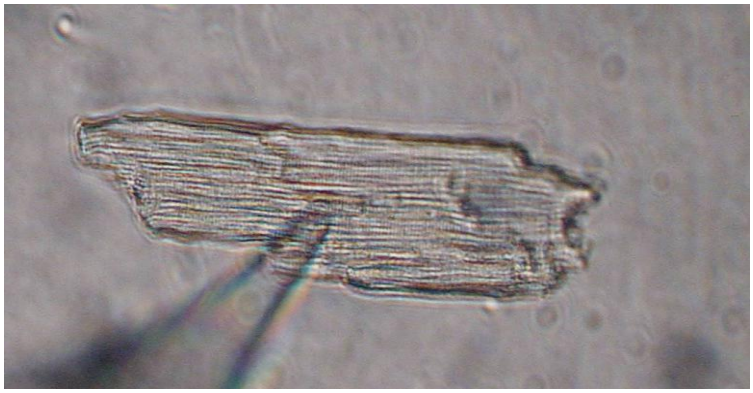


Figure 2.7: Rat ventricular myocyte with patch pipette

2.4.2 Experimental protocol

These studies were used to measure L-type calcium current ($I_{Ca,L}$) measurements and the I_{Na}. For the $I_{Ca,L}$ measurement the external solution was perfused at 34^{o} C. The I_{Na}

current was inactivated to be able to measure the much smaller $I_{Ca,L}$ using a step from -70 mV to -40 mV and the K^+ currents were abolished by replacing K^+ with Cs^+ in the pipette solution. $I_{Ca,L}$ (measured as the peak inward current) was elicited by a step depolarisation from -40 mV to voltages in the range -30 mV to +50 mV at a frequency of 5 Hz for 200 ms. For I_{Na} measurements, the external solution was superfused at 23°C only (it was not possible to maintain consistent seals for the length of the experiment at 34°C) (Figure 2.8). We used holding potentials of -90mV and the current was elicited by stepping to -50 mV at a frequency of 5 Hz for a 100 ms. This was after performing an IV relationship study (from -70 to +10 mV) and established the peak current at -50 mV. Currents were sampled at 10 kHz and filtered at 5 kHz.

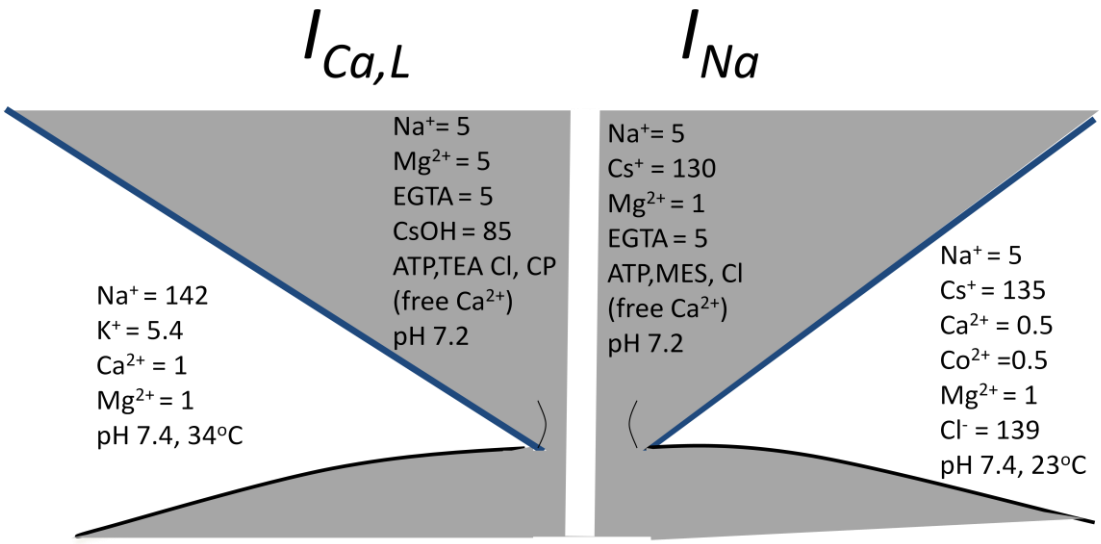


Figure 2.8: Schematic representation of the ruptured-patch technique showing the composition (in mM) of the internal and external solutions for the $I_{Ca,L}$ experiments (left) and the I_{Na} experiments (right).

2.5 Langendorff perfused rat heart experiments

2.5.1 Harvesting of hearts

Rats were anaesthetised using intraperitoneal injection of pentobarbitone sodium (60 mg/kg). The femoral vein was exposed and 50 IU of heparin was injected to prevent

thrombus formation in the microcirculation of the heart. The chest was then opened through a top-roof incision in the abdomen and the heart was exposed, excised by transecting the major vessels leaving a relatively long part of the aorta attached to the heart for perfusion and immediately immersed in ice-cold KHB solution (4^oC) to arrest and protect the heart. The heart is then attached to the perfusion cannula via the aorta and perfused with 95% O_2: 5% CO_2 gassed KHB at a temperature of 37^oC and controlled pressure of 100 cm of water within 30-60 seconds of harvest.

2.5.2 Langendorff perfusion system and setup

The isolated hearts are attached to a stainless steel cannula with circumferential grooves. The heart can be perfused with different solutions stored in elevated reservoirs providing a perfusion pressure of 100 cm of water. All glassware used were water jacketed for temperature control. The function of the heart is measured by inserting a water-filled balloon (constructed from cling film tied on the end of a catheter) inside the left ventricle. The balloon is connected to a pressure transducer and the pressure trace is recorded via a bridge amplifier using the Powerlab® system (ADI Instruments Chalgrove, Oxford, UK). Electrocardiogram (ECG) was also recorded using a silver wire connected to Powerlab® by a bio-amplifier. The balloon volume was adjusted during the stabilisation to achieve an end-diastolic pressure between 4-8 mmHg. The left ventricular developed pressure (LVDP) was electronically calculated from the difference between systolic and diastolic pressure. The heart rate was electronically calculated from the time of the cyclic change in the more stable pressure trace rather than the ECG signal which was prone to noise and sensitive to interference. However, ECG was always monitored to insure the lack of any electrical activity during arrest and to measure atrial activity during ventricular arrest and atrio-ventricular dissociation. The heart is kept in a water-jacketed glass reservoir covered with paraffin film ‘parafilm’ to maintain the temperature. The coronary perfusate was collected and the flow was calculated on a regular basis. Hearts were left to stabilise for 20 minutes with KHB perfusion at 37^oC, and they were included in the study if heart rate was between 200 and 400 bpm, LVDP between 100 and 180 mmHg and coronary perfusate between 10 and 16 ml/min.

2.6 **Statistical analysis**

Data are presented as mean ± standard error. Difference between means was analysed by paired *t*-tests or ANOVA with post-hoc Dunnet's test for multiple group comparison where appropriate. In the concentration-response graphs, the values were expressed as a percentage of control (no drug) and the sigmoid curves were fitted using GraphPad Prism software to the following equation: $Y= Y_{min} + (Y_{max}-Y_{min})/(1+(IC_{50}/X)^{-n})$ where X is the drug concentration, IC_{50} is the concentration for 50% of the maximal inhibition, and n is the Hill coefficient

CHAPTER 3. RESULTS 1: THE NEGATIVE INOTROPIC EFFECT OF ESMOLOL IN THE ISOLATED MYOCYTES

3.1 Introduction

We (Bessho and Chambers 2001) previously showed that esmolol at (a concentration of 0.3 mM) had a significant inhibitory effect on contraction (Figure 1.13), with a 75% decrease in LVDP. The effect on heart rate, however, was less dramatic with only a 20% decrease. Clinically, esmolol has been used in combination with blood-based preparation as an alternative to conventional cardioplegic arrest with the crystalloid Bretschneider solution (Mehlhorn et al. 1999) or with blood hyperkalaemia (Scorsin et al. 2003). Both studies reported that esmolol significantly inhibited myocardial contraction with an initial infusion concentration estimated to be around 0.4-0.7 mM directly into the coronary arteries, which allowed them to operate on the heart despite the fact that the heart rate was maintained at around 30-45 bpm. The negative inotropic effect of esmolol was presumed to be due to its β-blocking action; however, it is unlikely that the β-blocking effect can induce such profound negative inotropy. This hypothesis is confirmed by Bessho's findings (Bessho and Chambers 2001) (Figure 1.13) where this negative inotropic effect was demonstrated in Langendorff perfused hearts with no catecholamine background. Additionally, the pilot data demonstrated that esmolol at 1mM induced arrest in the paced isolated ventricular myocyte where again, there is no background adrenergic stimulation. This effect was not observed with the β-blocker atenolol at the same concentration of 1 mM (Figure 1.14). This pilot data also demonstrated that the calcium transient (Ca_{tr}) decreased by about 50%, which would explain the profound negative inotropic effect observed in the experimental studies by Bessho and the clinical studies by Scorsin (Scorsin et al. 2003), Melhorn (Mehlhorn et al. 1999) and others (Kuhn-Regnier et al. 1999; Kuhn-Regnier et al. 2002). It is, however, difficult to understand the complete arrest by in our pilot experiment. Consequently, this chapter aims to examine in detail the negative inotropic and Ca_{tr} inhibitory effects of esmolol in the electrically stimulated isolated rat ventricular myocytes.

3.2 **Methods**

Experiments were conducted in isolated rat ventricular myocytes loaded with Fura-2, for the measurement of cell contraction and the calcium transient as detailed in Methods (Section 2.3). In all experiments, each myocyte was chosen to fit the inclusion criteria of having a rod shape with no membrane blebs and the diastolic sarcomere length (SL) between 1.7 and 2.0 µm while being superfused with Tyrode solution at the desired temperature in the perfusion bath. The sarcomere shortening (SS) and Ca_{tr} were measured for at least 10 seconds as control after a period of 90 seconds for stabilisation. Then, the treatment was applied at the desired concentration by switching the superfusion solution using the multiple channel manifold (see details in Section 2.3.3). After reaching the steady state (usually within 3 minutes of superfusion) the Ca_{tr} and the SS were measured again to study the effect. The desired treatment then was applied by switching the superfusion solution from Tyrode to the desired drug diluted in Tyrode solution at the desired concentration. To ensure that the observed effect is caused by the treatment and to determine the reversibility of the treatment, washout period with Tyrode solution for up to 5 minutes is conducted and SS and Ca_{tr} recorded again to demonstrate recovery. After this measurement is completed the myocytes in the bath were washed out thoroughly and another aliquot of fresh myocyte suspension was used.

3.3 **Results**

3.3.1 The negative inotropic effect of esmolol (1 mM)

The inotropic effect of esmolol was initially studied in the electrically-stimulated intact isolated rat ventricular myocyte to reproduce the effect demonstrated in the pilot data, without the fluorescence dye. In these experiments the excitation light from the arc lamp and the hyper-switch were turned off and IonOptix recorded only the SL.

Esmolol (1 mM) was superfused for 120 seconds at room temperature and the esmolol effect on SS was calculated from the last 10 seconds. Finally, the reversibility of esmolol was studied by superfusing the myocytes with Tyrode again for up to 5 minutes until reaching a steady state to allow adequate washout of esmolol and the washout effect on SS was calculated from the last 10 seconds of the recording. Nine myocytes were studied in these experiments (*n*=9). Esmolol (1 mM) had an extensive negative inotropic effect on contraction, as shown by a decrease of 86.8 % in SS from the control value of 0.19 ± 0.03 µm (mean ± SE) to 0.03 ± 0.01. SS partially recovered to 59% of control to 0.11 ± 0.03 µm (Figure 3.1). This suggested that the negative inotropic effect of esmolol demonstrated by our single experiment in the pilot data was reproducible. Interestingly, during the treatment with esmolol the threshold of excitation in order to generate contraction became higher and occasionally the pacing voltage had to be increased significantly in order to induce excitation and contraction. We observed this in all the following paced isolated myocytes studies later. The underlying mechanism of this incidental finding was later explained in Chapter 7. However, the next step was to establish the effect of esmolol on the calcium transient in conjunction with its effect on contraction

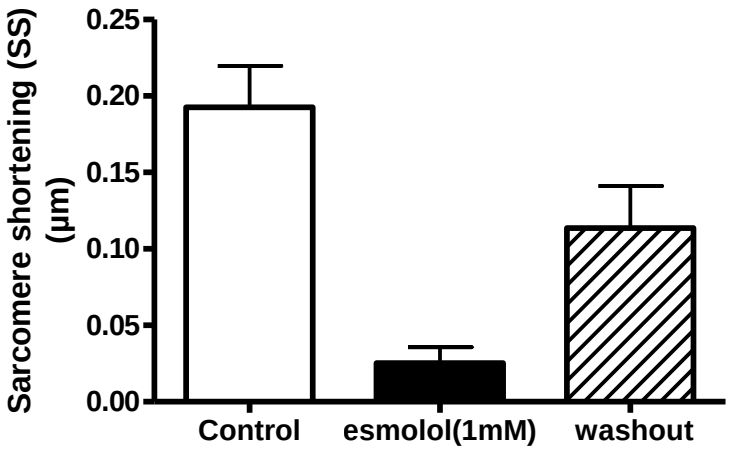


Figure 3.1: The effect of esmolol on contraction in the electrically stimulated intact isolated rat ventricular myocytes. This effect is partially reversible in the washout period *n=9*.

3.3.2 Effect of esmolol (1 mM) on Ca_{tr} and SS

After establishing the negative inotropic effect of esmolol (1 mM) in the isolated ventricular myocytes, these experiments were performed to reproduce the effect of esmolol (1 mM) on SS and Ca_{tr} using fura-2-loaded myocytes. This was previously demonstrated in our pilot data using different Ca^{2+} sensitive dye, Indo-1. Esmolol had the same negative inotropic effect on contraction within a few seconds of introduction. Esmolol also decreased the intracellular calcium Ca_{tr} (Figure 3.2,Figure 3.3, Figure 3.4). Esmolol (1 mM) made a few cells completely unexcitable despite using maximum voltage potentials (40 volts), Ca_{tr} was fully abolished (Figure 3.2); However, most cells exhibited a partial negative effect on contraction and Ca_{tr} (Figure 3.3Figure 3.4). In the pilot data, complete arrest in contraction was induced by esmolol (1 mM), but the Ca_{tr} was not completely abolished; this was different to the present results, where esmolol arrest completely abolished Ca_{tr} (Figure 3.2).

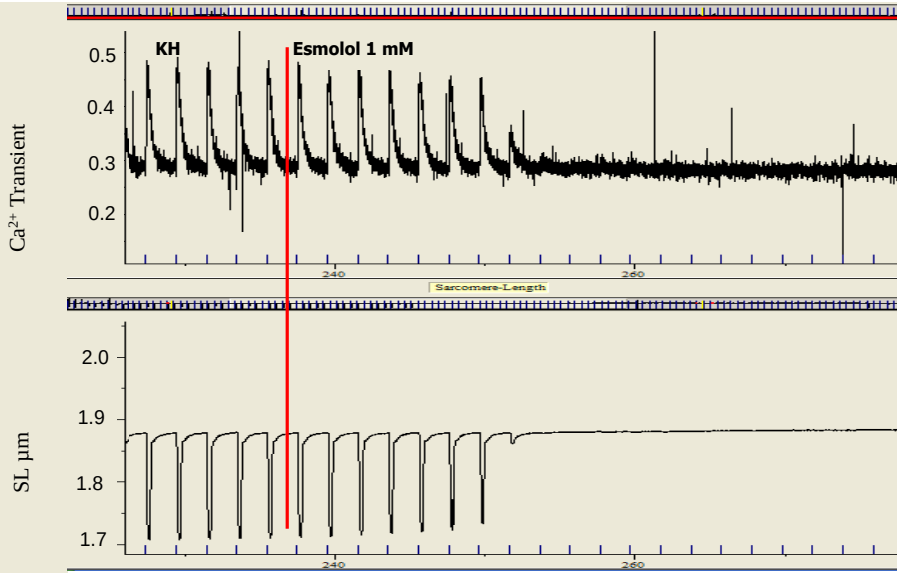


Figure 3.2: Example of myocyte treated with esmolol (1mM) when complete arrest was induced.

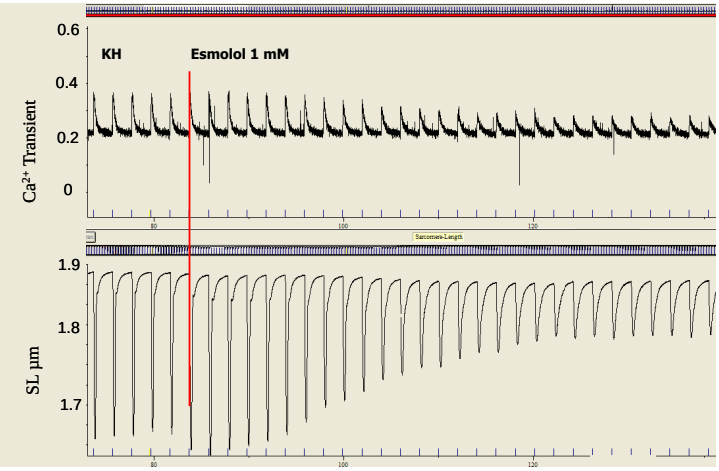


Figure 3.3: Example of myocyte treated with esmolol (1mM) with negative effect on contraction and Ca_{tr} without inducing full arrest.

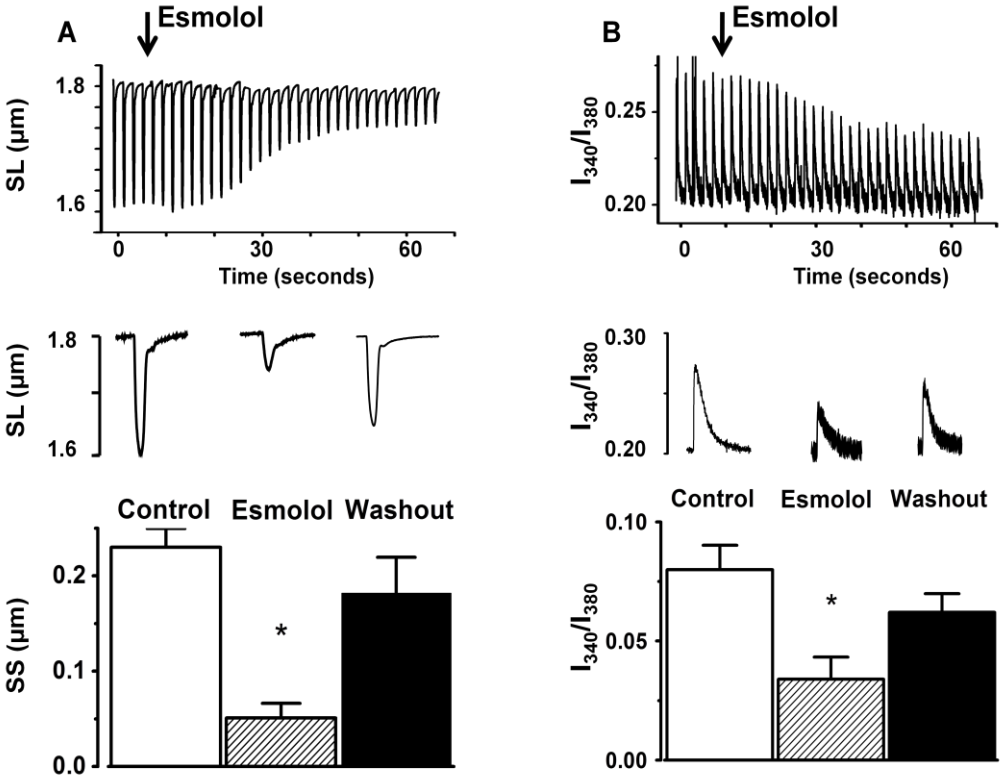


Figure 3.4: The negative inotropic effect of esmolol (1 mM). Effect on sarcomere shortening (A) and the fura-2 fluorescence ratio (Ca_{tr}), (B). Top panels: typical chart records showing the rapid fall of contraction. Middle panels: example contractions and Ca^{2+} transients. Lower panels: averaged data for the amplitudes of sarcomere shortening and the Ca^{2+} transient (*n*=5/ group). * *P*<0.01 *vs*. control.

3.3.3 Effect of esmolol on ionised calcium

The previous experiments suggested that esmolol inhibited contraction by inhibiting Ca_{tr}. However, an alternative possibility is that esmolol, or another compound in the Brevibloc® preparation, could have chemically decreased the availability of ionised calcium in the perfusate by having a calcium chelating effect. Any calcium chelating effect will decrease ionised calcium in the extracellular space, which decreases the $I_{Ca,L}$ via the L-type calcium channel. This will subsequently decrease the availability of ionised calcium in the intracellular space to trigger the calcium-induced calcium-release from the SR, leading to a decrease in Ca_{tr} and contraction. It is therefore important to determine whether esmolol or any other compound in the Brevibloc® preparation has any chelating effect, which might explain these findings before performing further studies to establish, which cellular targets might be involved in this effect.

In order to investigate the chelating effect of Brevibloc®, the ionised Ca^{2+} concentration $[Ca^{2+}]$ was measured in both Tyrode (1mM $CaCl_2$) solution and Tyrode + Brevibloc® at an esmolol concentration of 1mM using a blood-gas analyser. The $[Ca^{2+}]$ was 1.15±0.2 mM in the Tyrode solution compared with 1.23±0.2 in the Tyrode + Brevibloc®. The difference in $[Ca^{2+}]$ in the 2 solutions was not significant, which strongly suggests that the inhibitory effect of esmolol on the calcium transient demonstrated above is not achieved by directly chelating extracellular ionised calcium.

3.3.4 Esmolol vs. atenolol

It was important to determine whether the effects seen with esmolol were common to other β-blockers. Hence, we conducted experiments in, which investigated the effect of another β blocker (atenolol) on the Ca_{tr} and SS. In addition, the effect of esmolol was studied in the presence of atenolol, when the β-receptors should be blocked and the effect of esmolol should be altered if it was mediated by the β-receptors. As above, intact isolated rat ventricular myocytes loaded with fura-2 were superfused with Tyrode solution for a 90 seconds stabilisation period and the control values of Ca_{tr} and SS were calculated from the last 10 seconds. Subsequently, atenolol (1 mM) in Tyrode solution was perfused for 5 minutes to ensure the recording of any delayed effect of atenolol (in

a separate pilot study atenolol had no effect if the cells were treated with atenolol for over 15 minutes). Then, on the background of continuous atenolol perfusion, 1 mM esmolol was perfused in the myocyte bath for at least a further 2 minutes after reaching the steady state. Figure 3.5 shows an example trace of the effect of atenolol alone and esmolol + atenolol. The myocyte SS during treatment with atenolol (0.26±0.04 µm) was not significantly different from the control SS (0.24±0.04 µm *P=0.12*), while with atenolol + esmolol, the SS decreased by 65% to 0.09±0.02 µm (*P<0.0001)*. For the Ca_{tr}, the control and atenolol values were not significantly different (0.11 ± 0.04 and 0.12 ± 0.04 respectively, *P=0.12),* while Ca_{tr} dropped by 50% with atenolol+esmolol to 0.06 ± 0.03 (*P<0.0001)* (Figure 3.6).

These results demonstrate that the effect of esmolol on Ca_{tr} and SS is not shared or altered by another β-blocker, atenolol; this strongly suggests that the negative inotropic effect of esmolol does not require functioning β receptors.

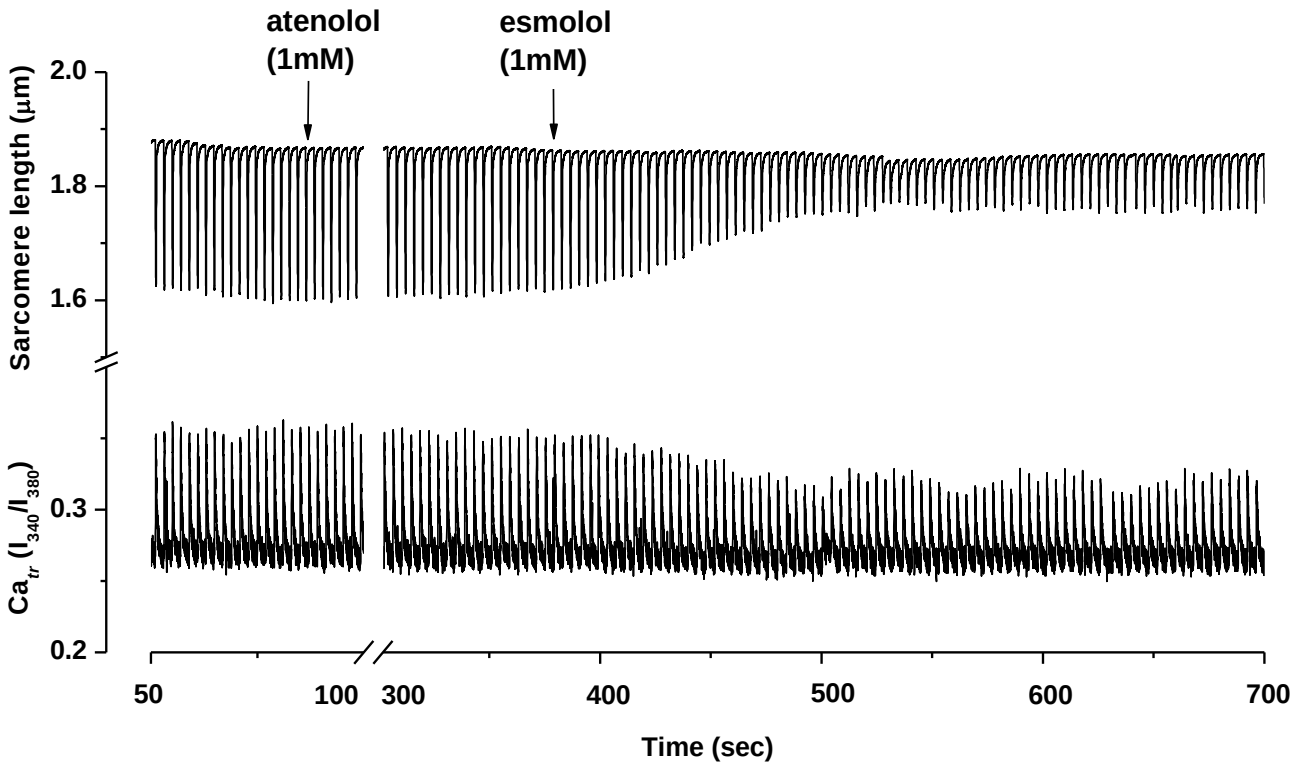
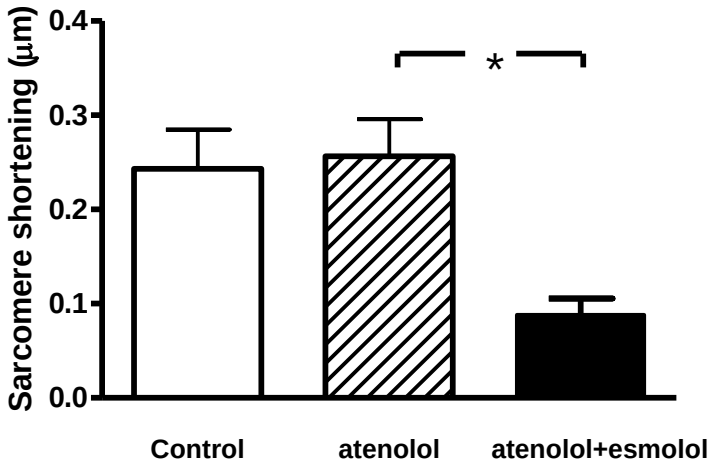


Figure 3.5: Example trace of the lack of β1-adrenoceptor participation in the effect of esmolol. Atenolol (1 mM) had no effect on SS (top) or Ca_{tr} (bottom). Meanwhile adding esmolol (1mM) inhibited contraction and Ca_{tr}.

A



B

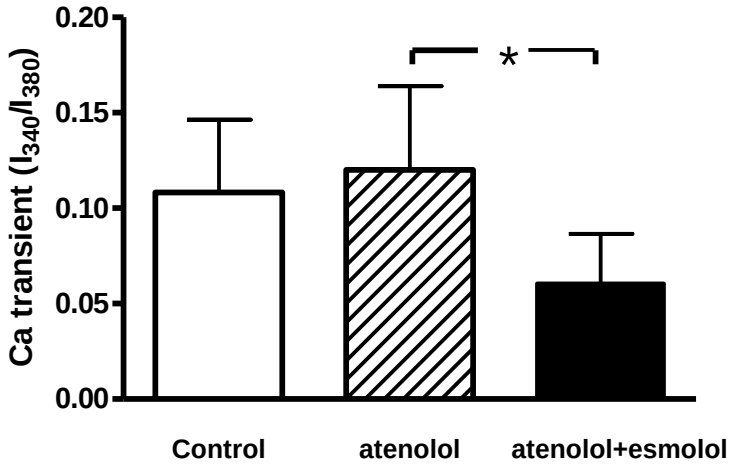


Figure 3.6: Lack of β1-adrenoceptor participation in the effect of esmolol on A: Sarcomere shortening and B: Calcium transient. The effect of atenolol (1 mM) vs. atenolol + esmolol (both 1 mM). Control was with no drug added. * P<0.0001 (n=5/ group).

3.3.5 The concentration-response curve of the effect of esmolol on isolated cardiac myocytes at 23^{o} C

Having established that the observed effects of esmolol on SS and Ca_{tr} were not caused by calcium chelation or a general characteristic of β-blockers, we were interested to determine the concentration-response effects in isolated myocytes. The studies were conducted at 23^{o} C (room temperature) as this temperature would be used clinically for cardioplegic solution. We also investigated this effect at 34^{o} C as this is close to physiological normal temperature. The experiments were performed to determine the millimolar concentrations-response relation for both contractility and the Ca_{tr}. We used 4 concentrations for these experiments: 0.03, 0.1, 0.3 and 1 mM. The fura-2 loaded myocytes were perfused as above for the 90 second-stabilisation period with Tyrode and control Ca_{tr} and SS were measured, then each cell was randomised into one of the four above mentioned groups of different concentration of esmolol and perfused for 2 minutes. A single cell per concentration was studied with a fresh cell suspension was used every time to ensure that each cell was only treated with a single concentration of esmolol. To measure the reversibility from esmolol inhibition, SS and Ca_{tr} were measured after washing esmolol for 3 minutes in Tyrode (recovery). Five cells were included in each group; Ca_{tr} and SS were normalised to the control value. The data shows that, with increasing esmolol concentrations, there is a progressive decline in both SS and Ca_{tr} (Figure 3.7); The data is plotted as a concentration response curve with the best sigmoid fit (using Prism® statistical analysis) using the equation mentioned in the Method Section 2.6. From this curve the IC_{50} for the SS was 0.13±0.04 mM. (Hill coefficient = -1.6 ± 0.6) while the IC_{50} of Ca_{tr} was 0.16±0.02 mM. (Hill coefficient = -1.3± 0.19).

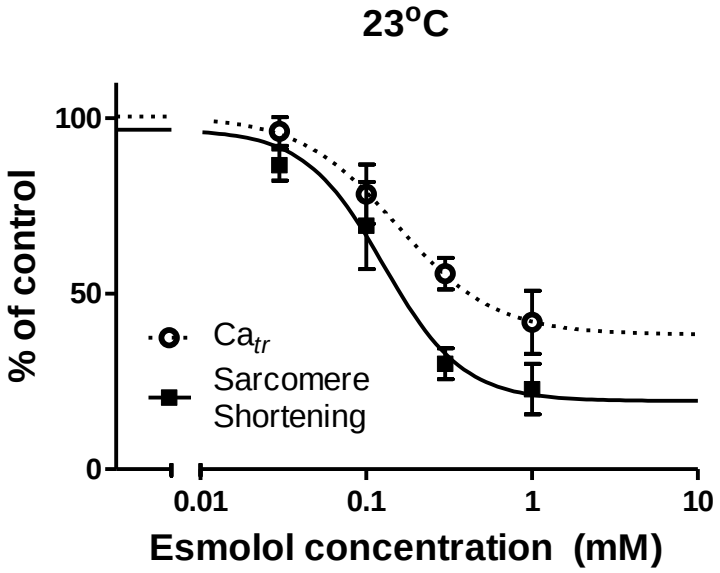


Figure 3.7: Concentration-response curves. The effect of esmolol on contraction and calcium transients at 23° C (*n*=5/group)

3.3.6 The concentration-response curve of the effect of esmolol on isolated cardiac myocytes in 34° C

The negative effect of esmolol on contraction and Ca_{tr} was demonstrated at 23°C earlier; this is a preferred temperature used for cardioplegia (the main application of esmolol at millimolar concentrations). However, it is essential to investigate whether esmolol will maintain the same effect in more physiological temperature. At 37°C, the myocytes tend to die more quickly, therefore 34°C was selected to be used as a temperature close to physiological and similar experiments to those described earlier were repeated at 34°C.

Esmolol had a comparable negative effect on contraction and Ca_{tr} at 23°C and 34°C. A sigmoid concentration-response curve was constructed (Figure 3.8) and the IC_{50} for SS was 0.16±0.02 mM (Hill coefficient = -1.04±0.06) while the IC_{50} of Ca_{tr} was 0.24±0.05mM (Hill coefficient =-1.14±0.2).

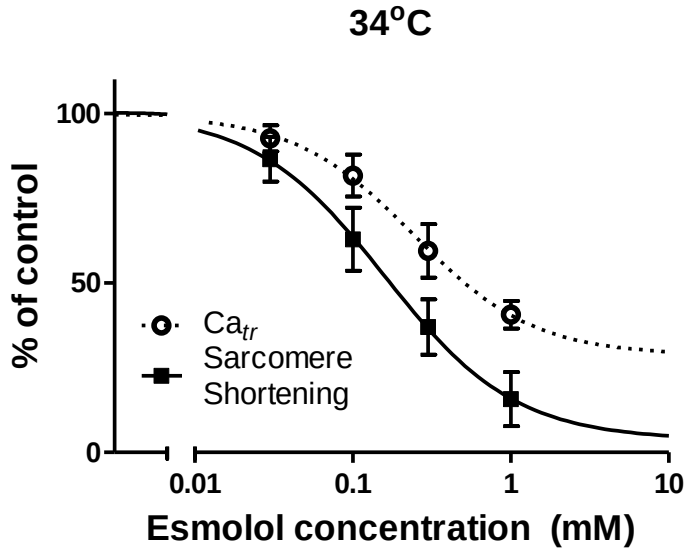


Figure 3.8: Concentration-response curves. The effect of esmolol on contraction and calcium transients at 34° C (*n*=5/group)

3.4 **Discussion**

Esmolol as highly selective β1-blocker is expected to have a negative inotropic and chronotropic effect. This effect was assumed to be the mechanism behind the "minimal myocardial contraction" in the clinical studies, which used esmolol as an alternative to conventional cardioplegia. However, any possible effect of esmolol as a beta blocker in the isolated hearts is likely to be negligible due to the nature of the sympathetic innervation of the heart (Kuhn-Regnier et al. 1999; Mehlhorn et al. 1999; Kuhn-Regnier et al. 2002; Scorsin et al. 2003); in the Langendorff rat hearts for instance (Bessho and Chambers 2001; Bessho and Chambers 2002) any possible adrenaline to be counteracted by esmolol would have been through the blood stream via coronary circulation from the adrenal glands (*Drake* 2005) On the other hand the less relevant catecholamine, noradrenalin, is mainly an Alfa receptor agonist with minimal direct effect on contraction. Noradrenalin is released in the heart by the axon endings of the thoracolumbar chain neurons rather than within the heart itself which makes a Langendorff perfused heart very low in catecholamine contents and by perfusing these

hearts during the stabilisation period most of these catecholamine compounds would have been washout out. Therefore esmolol is likely to have exerted its effect independently from any other catecholamine in the heart. The profound negative inotropic effect achieved by esmolol in these studies was much more than the expected effect of a β-blocker and suggests that esmolol had a direct inhibitory effect on contraction in addition to reducing heart rate to 30-40 bpm in these patients. As shown in Section 3.3.4, esmolol induced its negative inotropic action on the electrically stimulated rat ventricular myocyte despite a background infusion of the other β1-blocker, atenolol which seems to suggest that this effect is not related to the β1-receptor. Some non-selective β-blockers could act directly as inverse agonists (Baker et al. 2003); however, this tends to occur at much lower concentrations (nano-μM) (Harding SE 2004). Additionally, esmolol, like atenolol, is a very selective β1-receptor antagonist and there is no evidence for inverse agonist effect in β1-receptors. Furthermore, an inverse agonist effect does not explain the complete abolition of contraction (Figure 3.2) or the reduced excitability, which resulted in the increased threshold of excitation mentioned in Section 3.3.1. Therefore, these findings confirm that esmolol has a direct inhibitory effect on contraction. There is also an inhibitory effect on the Ca_{tr}, which can wholly or partially explain this negative effect. However, the inhibition of Ca_{tr} seems to be less prominent than that on contraction as shown in Figures 3.7 and 3.8. It is well known that the relationship between Ca_{tr} and contraction is not linear (Bers 2002); however, discrepancy may still indicate that the myofilament sensitivity to intracellular calcium might have been reduced by esmolol. In particular, the pilot data (Figure 1.14) also showed almost complete abolition of contraction with esmolol, but only partial reduction of Ca_{tr} after esmolol superfusion. It was important, therefore, that we investigated whether esmolol acts by reducing the myofilament sensitivity as part of its negative inotropic effect. The studies in the following two chapters are aimed at exploring the mechanism of Ca_{tr} reduction and any possible effect of myofilament desensitising effect of esmolol.

3.5 **Summary and Key Findings**

We have demonstrated that esmolol inhibited contraction in the isolated paced myocytes. This effect was maintained with a background treatment of atenolol.

Subsequently, we loaded the myocytes with Fura 2 and esmolol inhibited contraction and the calcium transient in a dose dependent manner and maintained in both room temperature and more physiological (34) temperature. We also excluded any possible chelating effect to calcium esmolol might have had.

CHAPTER 4. RESULTS 2: THE EFFECT OF ESMOLOL ON THE SARCOPLASMIC RETICILUM

4.1 Introduction

In the previous chapter, we demonstrated that, in isolated cardiac myocytes, esmolol superfusion inhibited contraction by reducing sarcomere shortening (SS) and intracellular calcium transient (Ca_{tr}). As explained in Sections 1.5.3 and 1.5.5, the rise of intracellular calcium during the excitation-contraction coupling takes place at two levels: initially, entry of extracellular calcium via the activated L-type calcium channels occurs during the plateau phase of the action potential (Bers 1991; Bers 2002), and this leads to intracellular calcium-induced calcium-release from its storage in the sarcoplasmic reticulum (SR) (Bers 1991; Bers 2002). Other β-blockers like propranolol were shown to inhibit the uptake of calcium by the SR (Hess et al. 1968). This suggests possible effect of esmolol on the SR, which results in less intracellular release of the calcium during excitation. The aim of these studies was to determine whether esmolol exerted any effect on the SR. In order to investigate this, we studied the negative inotropic effect and the Ca_{tr} of esmolol in the isolated ventricular myocytes after chemically inhibiting the SR with Thapsigargin pre-treatment. If the SR is involved in the effect of esmolol this inhibitory effect would be modified.

4.2 Methods

After isolation rat ventricular myocytes (Section 2.3.1), they were treated for 10 minutes with the SR blocker thapsigargin (1μM) diluted in Dimethyl sulfoxide (DMSO). This will result in irreversible inhibition of the calcium release from the SR (Kijima et al. 1991). It also inhibits the uptake of calcium via the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) (Kijima et al. 1991; Lytton et al. 1991). The myocytes were loaded with fura-2 in a similar manner explained in the previous chapter. Once again the effect of esmolol on contraction and Ca_{tr} was studied by electrically stimulating these

myocytes and measuring contraction and Ca_{tr} using IonOptix system (Section 2.3.5). Provisional experiments were conducted to exclude any unexpected effect of the solvent DMSO by treating myocytes with DMSO only for 10 min before washing. Myocytes pre-treated with DMSO showed no alteration in the calcium transient or the contraction. Meanwhile, thapsigargin pre-treated myocytes had a profound negative inotropic effect and extremely reduced Ca_{tr} as expected. To overcome the large negative inotropic effect caused by blocking the SR, the extracellular calcium in the Tyrode solution was increased to 7 mM as this was found to be the concentration required to restore the contraction to normal values in cells with intact SR, which is around 0.2µm of sarcomere shortening. During this experiment, extra care was taken to insure that the calcium was desolved in the solution. In addition to blocking the calcium-induced calcium-release, thapsigargin also blocks the SERCA (Kijima et al. 1991; Lytton et al. 1991), which is responsible for the uptake of the free intracellular Ca^{2+} at the end of plateau phase. This meant that the drop in calcium will be much longer, which results in a prolonged plateau phase and, therefore, prolonged action potential in these myocytes. The high calcium entered into the cells during the excitation process was dependent entirely in this situation on the Na/Ca exchanger. Therefore, the cells were paced at 0.1 Hz instead of 1 Hz to allow enough time for the Na/Ca exchanger to remove the intracellular calcium and prevent tetanisation of the myocytes. With the increased extracellular [Ca^{2+}] in the superfusate and the reduced pacing rate, the calcium transient were measured the in the isolated rat ventricular myocytes loaded with Fura-2 (Section 2.3 above. The response to esmolol in thapsigargin treated group was compared with myocytes with intact SR, which were superfused as described in the last chapter with extra cellular Ca^{2+} of 1 mM in the superfusate of KHB followed by KHB + esmolol (0.3mM) and paced at 1 Hz. In brief, Myocytes were selected to fit the inclusion criteria of having a rod shape with no membrane blebs and the diastolic sarcomere length (SL) between 1.7 and 2.0 µm while being superfused with Tyrode solution at 23^{o}C in the perfusion bath. At least 5 contractions were used to calculate the average value of contraction presented by the sarcomere shortening (SS) and Ca_{tr} at every stage, pre-treatment baseline, contraction, and recovery.

4.3 **Results**

4.3.1 The effect of esmolol on cardiac myocytes with blocked SR

A single dose of esmolol (0.3mM) was chosen because it caused a decline in Ca_{tr} and contraction of around 40-60% of the control value (from previous experiments). The effect of esmolol in the myocytes with the blocked SR was compared with its effect in the intact SR myocytes, which were superfused with Tyrode solution containing 1 mM of extracellular calcium in a similar experimental conditions to those used in the previous chapter. Again the SS and Ca_{tr} during esmolol treatment period were normalised to the pre-treatment control value. The effect of esmolol shown previously in the intact myocytes was maintained in the myocytes treated with thapsigargin (Figure 4.1). Esmolol (0.3 mM) reduced SS in the intact SR group to 32±4 % of control compared with 39±8% in the blocked SR group. It also decreased Ca_{tr} to 57±4 % in the intact SR group compared with 56±8% (Figure 4.2). The effect of esmolol in the two groups on both SS and Ca_{tr} was not statistically significantly different using unpaired *t*-test (p=0.3 and 0.8 for Ca_{tr} and SS, respectively).

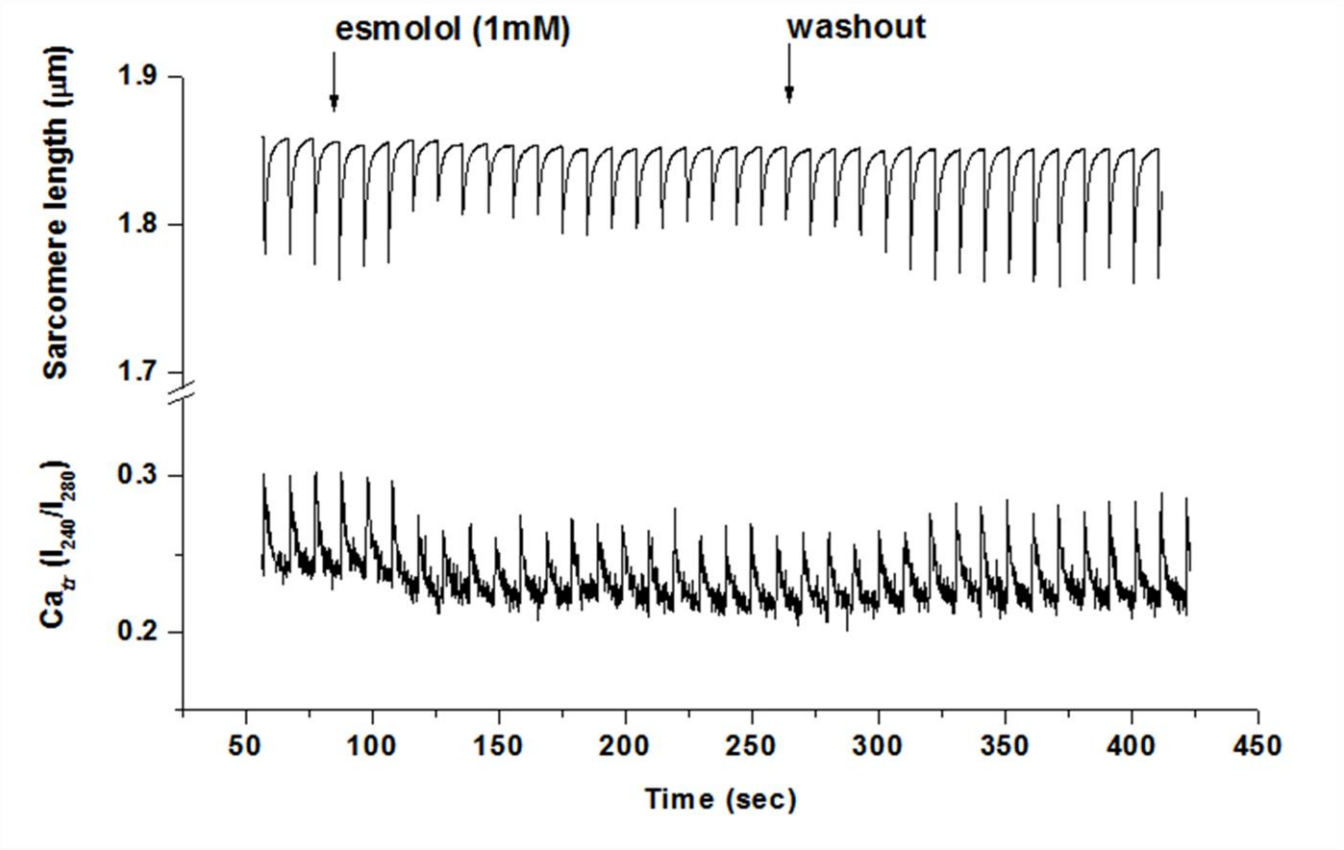
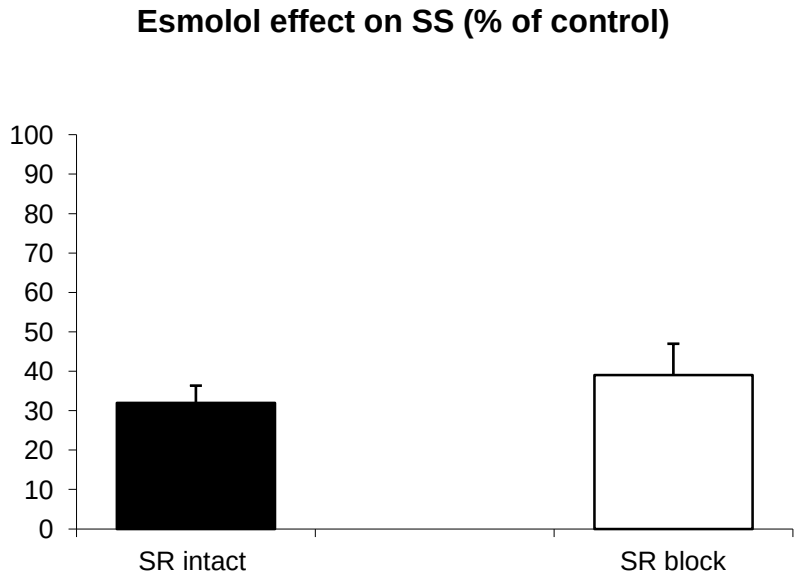


Figure 4.1: Example trace of the lack of the sarcoplasmic reticulum participation in the effect of esmolol. (0.3mM) inhibited contraction and Ca_{tr} in isolated rat ventricular myocyte treated with thapsigargin. The control contraction and Ca_{tr} were restored by increasing the extracellular calcium to 7 mM and to avoid tetanising the cells, the pacing rate was decreased to 0.1 Hz.



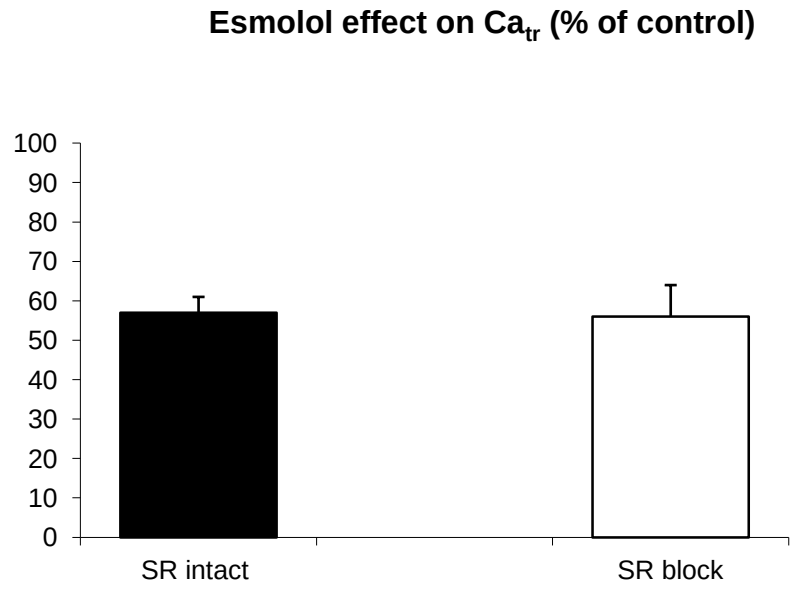


Figure 4.2: The role of the SR in the effect of esmolol. Inhibitory effect of esmolol 0.3 mM on contraction and calcium transient in myocytes with functional SR or myocytes treated with 1 μM thapsigargin (*n*=5/group). All data expressed relative to pre-esmolol (control) values in each group.

4.4 Discussion

As demonstrated above, the inhibitory effect of esmolol on contraction and the Ca_{tr} was maintained in the myocytes with inhibited SR. It was also comparable to the effect in the myocytes with intact SR. Other β-blockers were shown to inhibit the SR uptake of calcium at high concentrations (atenolol 1mM) an effect was shared to a less extent with other alpha blockers (Hess et al. 1968) (Cantilina et al. 1993). This was thought to be by inhibiting the SERCA (Hess et al. 1968) (Cantilina et al. 1993). However, in these experiment the SERCA was already blocked, which was demonstrated by the delayed relaxation, which confirms that the inhibiting effect of Ca_{tr} is independent form the SR. Therefore, we can speculate that the L-type calcium channels are the likely target by which esmolol blocked the calcium entry to the cell or what is known as the $I_{Ca,L}$ current. The effect of esmolol on the L-type calcium channels will be studied in chapter 6. However, initially, it was important to determine whether esmolol influence myofilament sensitivity to calcium, the mechanism suggested by the pilot data (Figure 1.14) and the dose response relationship (Figures 3.7 and 3.8). This will be described next.

4.5 Summary and Key Findings

This Chapter aimed at identifying any possible involvement of the SR in the inhibitory effect of esmolol on the calcium transient. We demonstrated that this effect was maintained in SR depleted myocytes which suggests that the esmolol inhibitory effect on contraction and the calcium transient is not dependent on the SR.

CHAPTER 5. RESULTS 3: THE EFFECT OF ESMOLOL ON MYOFILAMENT SENSITIVITY TO CALCIUM

5.1 Introduction

The protective effect of chronic use of β-blockers against ischaemia was well established through different mechanisms (Harding SE 2004; Purek et al. 2006). However, recently long term treatment of β-blockers (bisoprolol) prior to ischaemic events was shown to restore the myofilament sensitivity to calcium and resistor myofilament contractility in the remote myocardium (Duncker et al. 2009). Meanwhile myofilament desensitisation with BDM was shown by us to improve the protective effect of polarised arrest against ischaemia in the cold storage (Snabaitis and Chambers 1999). This highlights the importance of investigating any possible effect esmolol might have on the myofilaments. Earlier studies described in Chapter three showed that esmolol decreased Ca_{tr} in a dose dependent manner and that this effect was not abolished by blocking the β-receptors with atenolol (Section 3.3.4). We also showed that the inhibition of contraction (SS) by esmolol was more profound compared with Ca_{tr} inhibition with the IC_{50} for contraction inhibition being 0.16±0.02 mM vs. 0.24±0.05 mM for inhibition of the Ca_{tr} (Sections 3.3.5 and 3.3.6). This can be explained by the non-linear relationship between the intracellular calcium concentration and contraction (Solaro and Rarick 1998; Bers 2002) but could also suggest that esmolol could have a direct effect on the myofilament's sensitivity to calcium. A desensitising effect could also explain the results obtained in our pilot data (Figure 1.14) where 1mM esmolol abolished contraction (measured using edge detection method in an isolated cardiac myocyte loaded with the calcium fluorescence dye Indo-2) without abolishing the calcium transient completely. This study, however, has an important limitation that it was only performed on one cell (n=1); these findings were not reproducible in our detailed studies described in Chapter three, where every time esmolol induced complete arrest in an isolated cardiac myocyte the calcium transient was also abolished. Any desensitisation effect, however, cannot be excluded despite the

inability to reproduce these preliminary findings. The aim of the experimental work in this chapter is to study the effect of esmolol on the myofilaments sensitivity to calcium.

5.2 Methods

5.2.1 The intact myocytes experiments

The gold standard method in assessing the myofilament sensitivity to calcium is the skinned myocytes. However, we developed this method to assess any possible desensitising effect of esmolol on the myofilaments to calcium in the intact myocytes, which offers more physiological conditions. The myofilament sensitivity to calcium was studied in the isolated rat ventricular myocytes using the preparation described earlier in Sections 2.3.4 and 2.3.5. In brief, the isolated myocytes were loaded with Fura-2 and placed in the superfusion bath. A myocyte, which fits within the inclusion criteria was selected. After a period of stability contraction and Ca_{tr} were recorded. Subsequently, we studied the relationship between the calcium transient (Ca_{tr}) and contraction (SS) by gradually reducing extra-cellular calcium through switching the superfusion solution from the usual Tyrode with [Ca^{2+}] of 1 mM to Tyrode solution with [Ca^{2+}] of 0.5 mM and then to 0.3 mM. This decline in extra-cellular calcium leads to gradual decrease in Ca_{tr} and subsequently contraction by having less available intracellular calcium to the myofilaments, which reduces contractions. This resulted in gradual reduction in contraction and Ca_{tr} to around 40-60% from baseline. This gradual decline is recorded generating multiple data points representing the Ca_{tr} /SS relationship in the untreated myocyte. Subsequently, extracellular calcium wass restored again with normal calcium (1mM)-Tyrode for a few minutes until reaching baseline. After a period of steady state, the same myocytes is then treated with Tyrode + esmolol 0.3 mM. The gradual decline in contraction and Ca_{tr} over 4 or five beats was again recorded. The data points, which represent the relationship between each Ca_{tr} value and SS after treatment of esmolol was again plotted and compared between the negative inotropic effect of declining extracellular calcium and the negative inotropic effect of esmolol. The experiment was repeated using four cells. The advantage of this technique is to explore any individual effect of esmolol on desensitisation in a physiological environment where all the organelles of the myocytes are intact. Meanwhile there were some limitations in this

technique as the decline in Ca_{tr} and SS was faster in the esmolol group, which resulted in fewer data points in the middle range of Ca_{tr} measurements. Equally, the inhibitory effect of esmolol 0.3 mM seemed to be more profound after exposing the myocyte to a period of hypocalcaemia.

5.2.2 The skinned myocytes experiments

The methods using skinned myocytes were explained in details in Sections 2.3.2, 2.3.6 and 2.3.7. To summarise, skinned myocyte model is the gold standard method to study the myofilament sensitivity to Ca^{2+}as it directly measures the relationship of $[Ca^{2+}]_i$ with contraction presented by Sarcomere Length (SL) without the cell membrane and therefore overcome the limitations mentioned above. However, one can argue that myofilaments interaction with calcium in isolation of other organism is not physiological and could miss out on any possible intracellular modification esmolol might have, which depends on other intracellular organelles. In order to mitigate for such limitations we performed two sets of experiments; the first set is to study any direct effect of esmolol on the myofilaments after crossing the cell membrane. Therefore esmolol 0.3 mM was added to the relaxing and activating solutions and the effect of perfusing the myofilament with these solutions were compared with myofilaments perfused with esmolol free relaxing and activating solution. The second experiment was performed to examine whether esmolol might instead alter myofibrillar Ca^{2+} sensitivity by post-translational modifications (e.g. phosphorylation) in the intact cell; therefore, we treated intact myocytes with esmolol (0.3 mM) for 10 minutes before skinning. Subsequently, the myocytes were skinned and then the myofilaments sensitivity to calcium was studied and compared with untreated skinned myocytes. The calcium sensitivity measurements was performed by suspending the skinned myocytes in a cytosol like relaxing solution and few drops were placed in the cell perfusion system. The perfusion system allowed a large number of cells to be screened without the need for measuring isometric contractile force. The Ca^{2+} sensitivity of myofibrils within the myocytes was then determined by recording SL in a skinned myocyte while cycling the superfusing solutions between relaxing solution and Ca^{2+}-containing activating solutions ($23^{o}C$) (Figure 5.1)

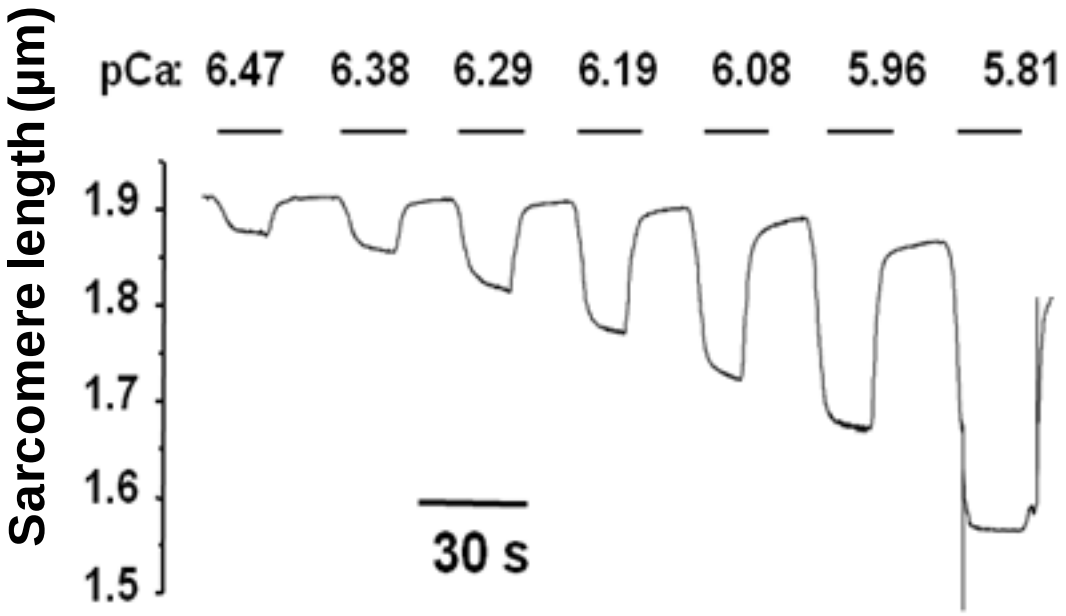


Figure 5.1 Experiments in skinned myocytes. Typical contraction responses of a skinned myocyte to Ca2+-containing activating solutions

5.3 Results

5.3.1 The effect of esmolol on myofilament sensitivity to calcium in intact myocytes

The relationship between sarcomere shortening and the Ca^{2+} transient amplitude in intact myocytes, determined by varying extracellular [Ca^{2+}], was compared with the SS/Ca_{tr} relationship determined during the negative inotropic effect of esmolol (Figure 5.2). Assuming that the negative inotropic effect of reducing extracellular [Ca^{2+}] results only from the fall of the Ca^{2+} transient, any difference between these shortening–[Ca^{2+}] relationships would indicate an additional action of esmolol (i.e. on the myofibrils directly). However, as Figure 5.2 illustrates, the data point plotted from Ca_{tr} /SS relationship generated by reducing extracellular [Ca^{2+}] fell over the data points of this relationship generated by superfusion with esmolol. Findings were observed in four other myocytes we slightly harder to interpret due to the lack of intermediate data points generated by esmolol but again there was no suggestion that esmolol had any additional desensitising effect. If esmolol had any desensitising effect in these experiments the

data points from esmolol superfusion would have shifted more to the right (Figure 5.2. Therefore we can conclude that much of the negative inotropic effect of esmolol may be explained by the depression of the Ca^{2+} transient.

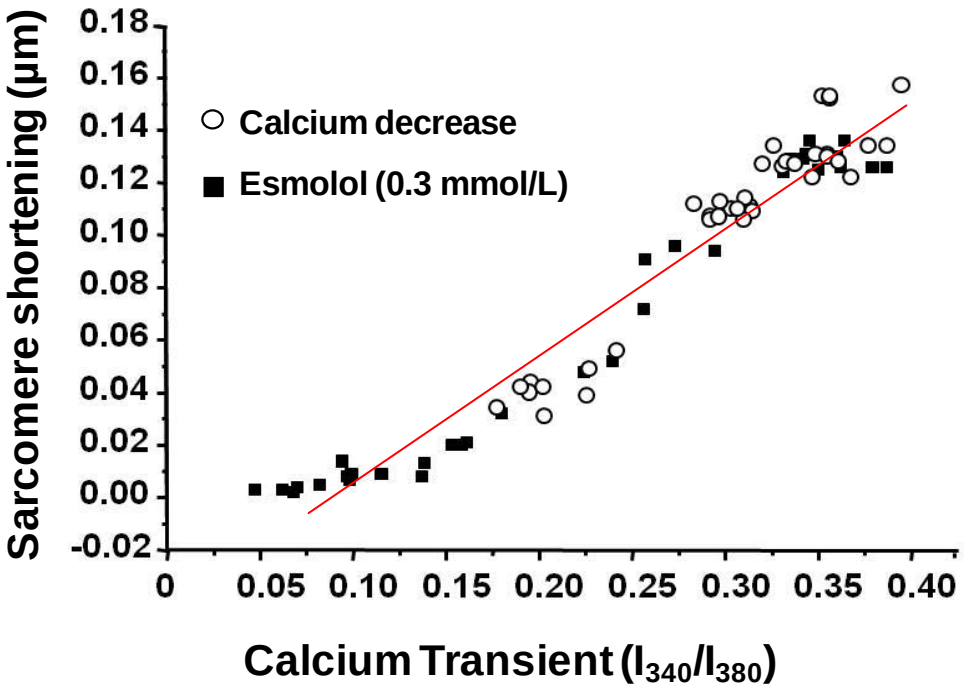


Figure 5.2 Effect of esmolol (0.3mM/L) on myofilament Ca^{+2}sensitivity. Experiment in intact myocytes. Results recorded from each contraction in a typical myocyte after decreasing the extracellular [Ca^{2+}] from 1 to 0.3 mM/L or adding 0.3 mM/L esmolol (*n*=5/group)

5.3.2 The Effect of esmolol on the myofilament sensitivity to calcium in the skinned myocytes

We, next, used skinned myocytes to establish whether esmolol has a direct effect on the myofilaments, without any contribution from other cellular organelles. We found that adding esmolol (0.3 mM/L) to relaxing and activating solutions had no direct effect on the Ca^{2+} sensitivity of myofibrils in the skinned myocytes (Figure 5.3). To examine whether esmolol might instead alter myofibrillar Ca^{2+} sensitivity by post-translational modifications in the intact cell, we treated intact myocytes with esmolol (0.3 mM/L) for

10 minutes and then skinned the myocytes. Comparison with a time-matched, control group of myocytes (i.e. not treated with esmolol before skinning) showed that pretreatment of the myocytes with esmolol before skinning did not alter the myofilaments' contractile response to Ca^{2+} (Figure 5.4). Thus, esmolol did not appear to alter myofibrillar Ca^{2+} sensitivity, suggesting that all of esmolol's inhibitory action results from the fall of the Ca^{2+} transient.

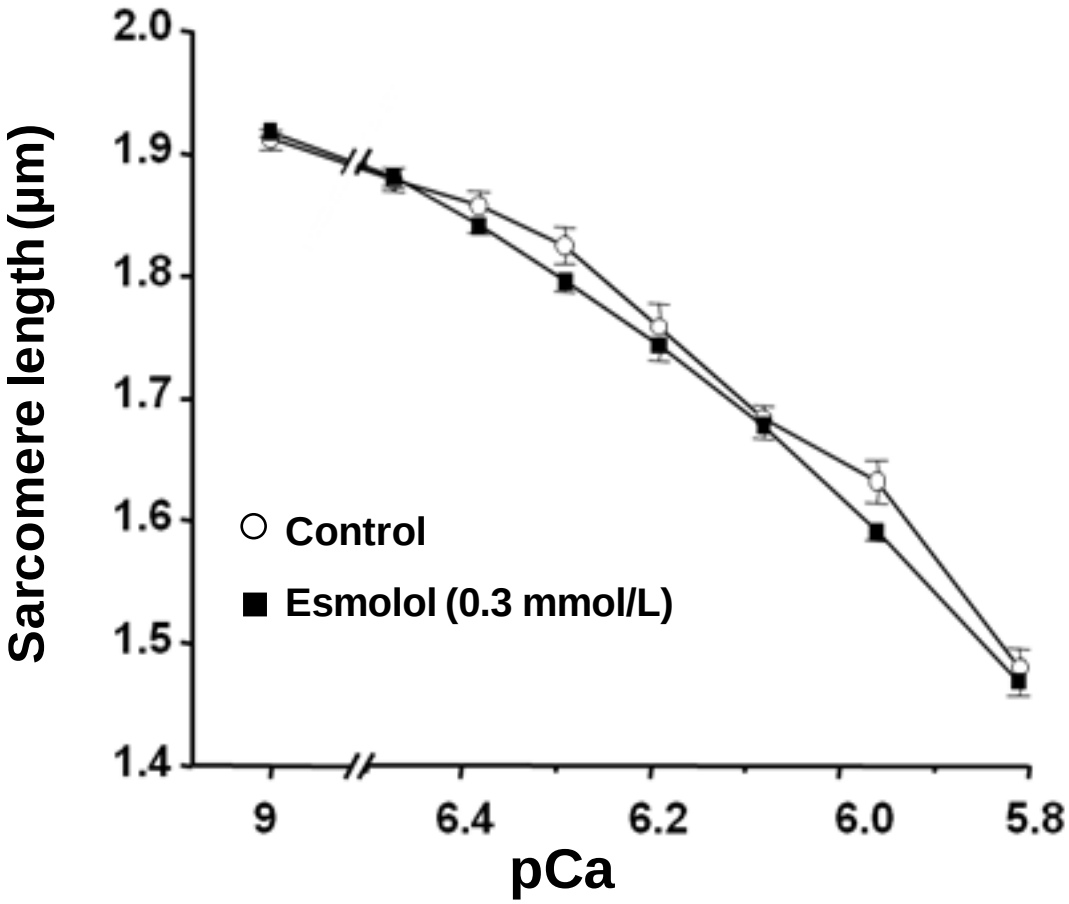


Figure 5.3 Experiments in skinned myocytes: The effect of superfusing the myofilaments with activating solutions with and without esmolol (n=9/group)

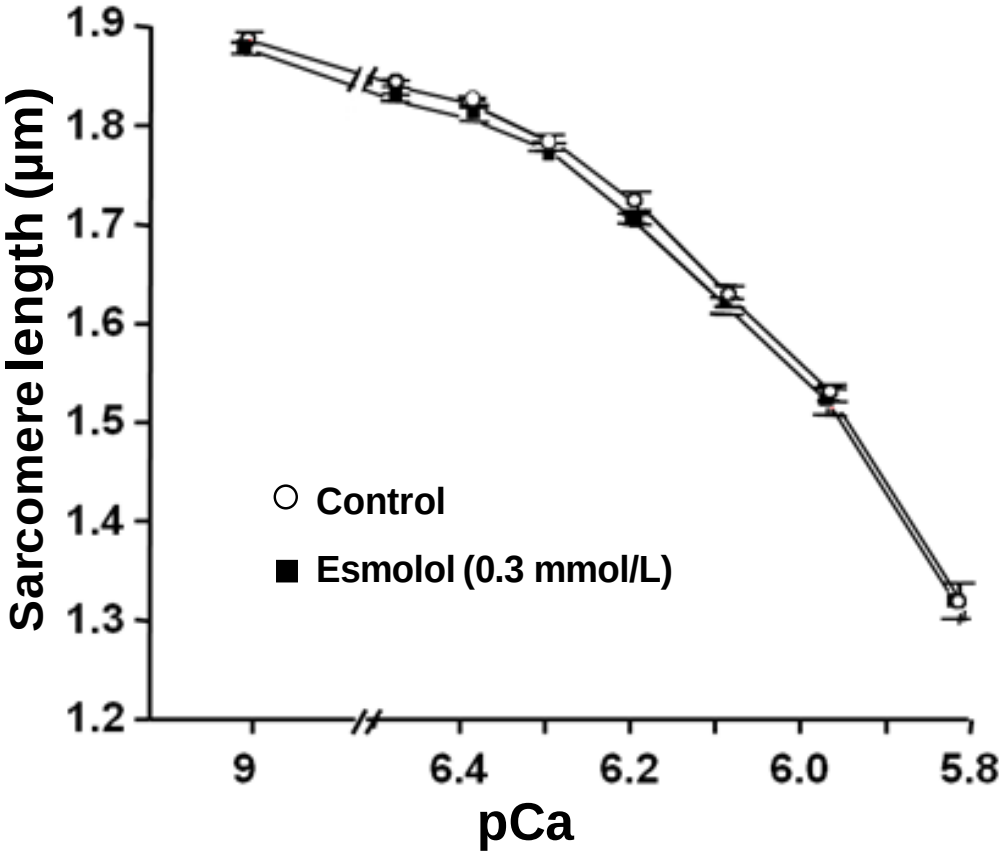


Figure 5.4: Experiments in skinned myocytes. The effect of pre-treating the myocytes with esmolol before skinning *vs.* control group. *n*=6/group (B).

5.4 **Discussion**

Failing myocardium is known by its deficiency in handling calcium (Lee and Terracciano 2010). Meanwhile, β-blockers (e.g. bisoprolol) were shown to have an effect on the myofilament remodelling in heart failure patients (Hall et al. 1995; Bristow 2000). Bisoprolol was, also, shown to improve myofilament sensitivity in the intact myocardium following MI in pigs on long term pre-ischaemic treatment (Duncker et al. 2009). However, there is no previous evidence, which shows that adrenergic receptor blockers can directly inhibit myofilament sensitivity to calcium. Our observation that contraction tends to be inhibited more than the Ca^{2+} transient (Figures 3.7 and 3.8), which suggested such effect may be explained by the fact that myofibrillar force

production is a non-linear function of intracellular [Ca^{2+}], such that small changes in [Ca^{2+}] translate into larger changes in contraction. On the other hand our pilot data, which was in a single myocyte were not reproducible. Therefore, these studies do not support the desensitising mechanism to explain the negative inotropic effect of esmolol. In the previous chapter, esmolol caused significant reduction in both, contraction and the Ca^{2+} transient in myocytes with inactive SR (Figure 4.1 Figure 4.2), which suggests that a direct effect of esmolol on the SR plays little or no part in its negative inotropic action. In these SR-inhibited myocytes, the Ca^{2+} transient is largely due to Ca^{2+} entry via the L-type calcium channel, since Ca^{2+} release from the SR is abolished. The large reduction in the Ca^{2+} transient under these conditions therefore suggested that a major action of esmolol was likely to be via inhibiting Ca^{2+} entry into the cell via the L-type Ca^{2+} -channels. Studies in the following chapter examine the effect of esmolol on the activity of the L-type calcium channels.

5.5 **Summary and Key Findings**

Alerting the sensitivity of the myofilaments to calcium was suggested as one of the mechanism in the pilot data. However, we demonstrated that the relationship of calcium and the sarcomere length was similar in the intact myocytes perfused with decreasing extracellular calcium and the esmolol-treated. We then explored this possible mechanism in the skinned myocytes and demonstrated that the myofilament sensitivity to calcium was not altered by either pre-treating the myocytes with esmolol or adding esmolol to the activation solution compared with the control group.

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CHAPTER 6. RESULTS 4: THE EFFECT OF ESMOLOL ON CALCIUM CURRENTS

6.1 Introduction

So far, we demonstrated that the negative inotropic effect of esmolol in isolated myocytes is independent from any β-blocking, and that the inhibition of contraction seems to be mainly due to the inhibition of the Ca_{tr} (Chapter 3). This proved that the profound negative inotropic effect observed in the clinical studies is different from the β-blocking effect assumed by Scorsin (Scorsin et al. 2003), Melhorn (Mehlhorn et al. 1999) and others (Kuhn-Regnier et al. 1999; Kuhn-Regnier et al. 2002). We have also excluded any possible involvement of the SR in this effect (Chapter 4) and despite some provisional evidence the possibility of any desensitisation to the myofilaments by esmolol was excluded using intact and skinned myocytes (Chapter 5). One mechanism, which might also explain the decline in Ca_{tr} by esmolol is that esmolol could have had a chelating effect on extracellular ionised calcium, resulting in a reduction in the ionised calcium availability and consequently reducing both calcium transient and the force of contraction. However, measurement of the ionised calcium concentration was found to be comparable in both Tyrode alone and the Tyrode +esmolol solutions. Therefore, the effect of esmolol should be through the cellular targets that handle the calcium transient and as explained previously esmolol is likely to have inhibited the entry of Ca^{2+} during stimulation via the L-type calcium channels. This effect was observed by a cardiac surgeon while performing an operation on a beating heart (Arlock et al. 2005). The author used esmolol to slightly slow the heart rate in order to be able to operate on the heart while beating in what is known as off-pump surgery. This resulted in an observed decrease inotropic function and sharp drop in blood pressure, which led his team to hypothesise that esmolol had a "direct negative inotropic effect on the myocardium". Steen and colleagues performed limited animal experiment in guinea pigs and pigs heart (Arlock et al. 2005); they measured the contraction of the paced ventricular trabecular muscle and the action potential after superfusion of esmolol (110 μM) and observed a

drop in the plateau phase of the action potential and the force of contraction. Moreover, they demonstrated a drop in $I_{Ca,L}$ in a single guinea pig myocyte patch clamping experiment (Arlock et al. 2005). These findings support the earlier hypothesis that the negative effect of esmolol on the calcium transient is by blocking the L-type calcium channels. This chapter aims to study the effect of esmolol on the L-type calcium channels in order confirm this hypothesis using the ruptured patch clamping technique.

6.2 **Methods**

Rat ventricular myocytes were isolated according to the method described in Section 2.3.1. A drop of the myocyte suspension was mounted in the perfusion chamber and the myocytes were allowed to settle on the glass bottom of the chamber for a minute. The myocytes were subsequently superfused with Tyrode solution. A hydraulic micromanipulator (MO-102, Narishige) was used to position the suction glass electrodes. The glass electrodes were manufactured from a pulled glass pipette filled with the appropriate internal solution and the resistance was measured while the tip is immersed in the bath solution. Pipettes with resistance between 1.5 and 3 MΩ were used to ensure appropriate tip diameter. A tight seal is formed between the tip of the pipette and the surface of the myocyte using the micromanipulators under X40 microscope objective lens. Seal resistance of greater than 1 GΩ was achieved by applying negative pressure (30-60 cm/H_2O). The patch membrane was then ruptured by a brief period of stronger suction. When a successful rupture was achieved with an access resistance less than 6 MΩ the $I_{Ca,L}$ current is measured while the cell is being superfused with Tyrode solution for the control measurement and the washout or with Tyrode + esmolol at the desired concentration.

$I_{Ca,L}$ was measured during superfusion with Tyrode's solution at 34^{o} C. Currents were sampled at 10 kHz and filtered at 5 kHz. pCLAMP 10 software (Axon Instruments) was used for the acquisition and analysis of currents. In order to be able to record the $I_{Ca,L}$, the very large current generated by the fast Na^+ channels was inactivated using a step from -70 mV to -40 mV and K^+ currents were abolished by replacing K^+ with Cs^+ in the internal solution. $I_{Ca,L}$ was elicited by a step depolarisation from -40 mV to voltages in the range -30 mV to +50 mV. The calcium current depicted in Figure 6.1 is the

negatively charged current required by the system to maintain the desired holding potential of the cell membrane. This current starts immediately after shifting the membrane potential and peaks around 25 ms. Subsequently it slowly decays when the inward calcium influx through the activated L-type calcium channels subsides, which could take as long as 150 to 200 ms. The size of this is current measured from the peak of this inward current to the steady state current after 200 ms. This method has been used by many authors as a well established method of measuring the $I_{Ca,L}$ (Liu and Kennedy 1998). As expected, this current was maximal at 0 to -10 mV holding potential. The concentration-response relationship was studied by superfusing the myocytes with Tyrode with increasing esmolol concentrations between 0.1 to 3 mM consecutively with 3 minutes superfusion time for each concentration. Thereafter, esmolol was washed out with Tyrode and $I_{Ca,L}$ was measured after 5 minutes to insure that the effect of esmolol is reversible. The effect of esmolol on the size of $I_{Ca,L}$ was normalised to the value before treatment as the control for each holding voltage. The sigmoid dose response curve was constructed using the effect of esmolol on $I_{Ca,L}$ at -10mV as it was resulted in maximum $I_{Ca,L,}$ the effect was presented in % of control value. Subsequently I/V relationship was studied in order to detect any possible effect of esmolol on the maximum activation of $I_{Ca,L}$ in relation to the membrane potential.

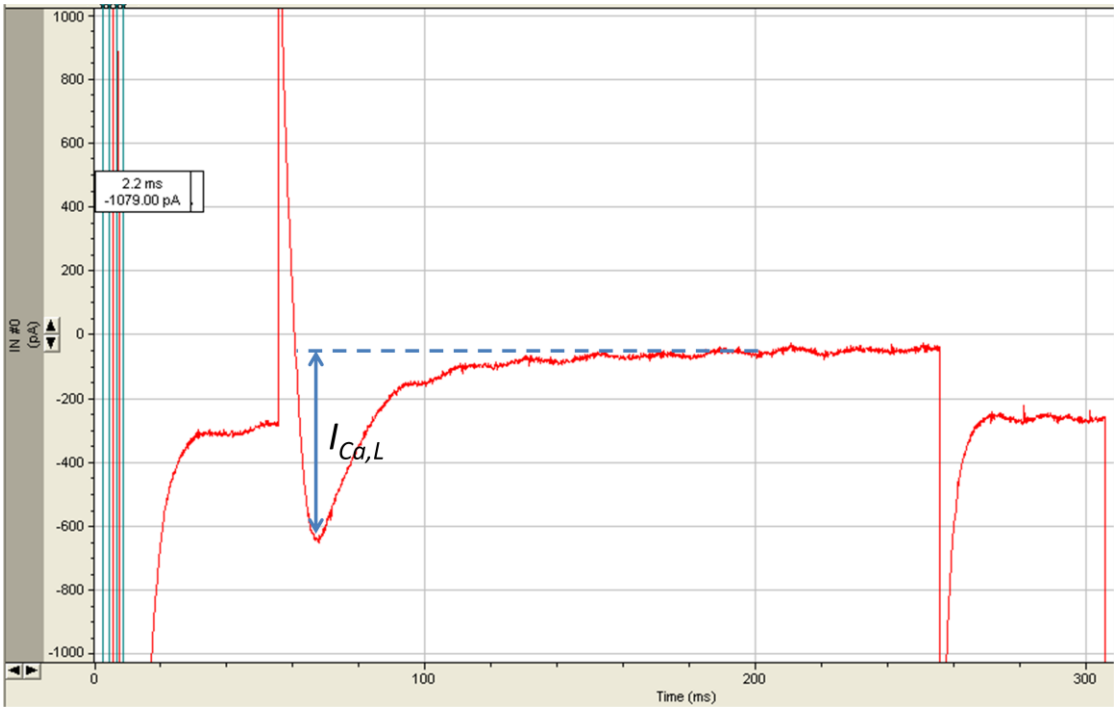


Figure 6.1 Typical recording of the calcium current demonstrating the method in calculating $I_{Ca,L}$

6.3 Results

6.3.1 Esmolol dose response curve on $I_{Ca,L}$

By treating the myocytes with stepping up concentrations of esmolol, it was found to have a dose dependent inhibitory effect on the $I_{Ca,L}$. The concentration-response relationship of this inhibitory effect on $I_{Ca,L}$ was studied by superfusing each myocyte with the increasing esmolol concentrations of 0.1, 0.3, 1.0 and 3.0 mM consecutively. At -10 mV, an esmolol concentration of 0.1 mM inhibited the $I_{Ca,L}$ by about 20%, while 3 mM almost abolished $I_{Ca,L.}$ This effect was partially reversible 5 minutes after washing out the drug. Figure 6.2 shows a typical recording of a cell demonstrating the inhibitory effect on $I_{Ca,L.}$ The concentration-response relationship curve was then constructed after normalising the effect of esmolol to control for each myocytes at -10 mV as shown in Figure 6.3. The mean IC_{50} in 5 myocytes was 0.45 ± 0.05 mM/L (Hill coefficient = 0.99 ± 0.18)

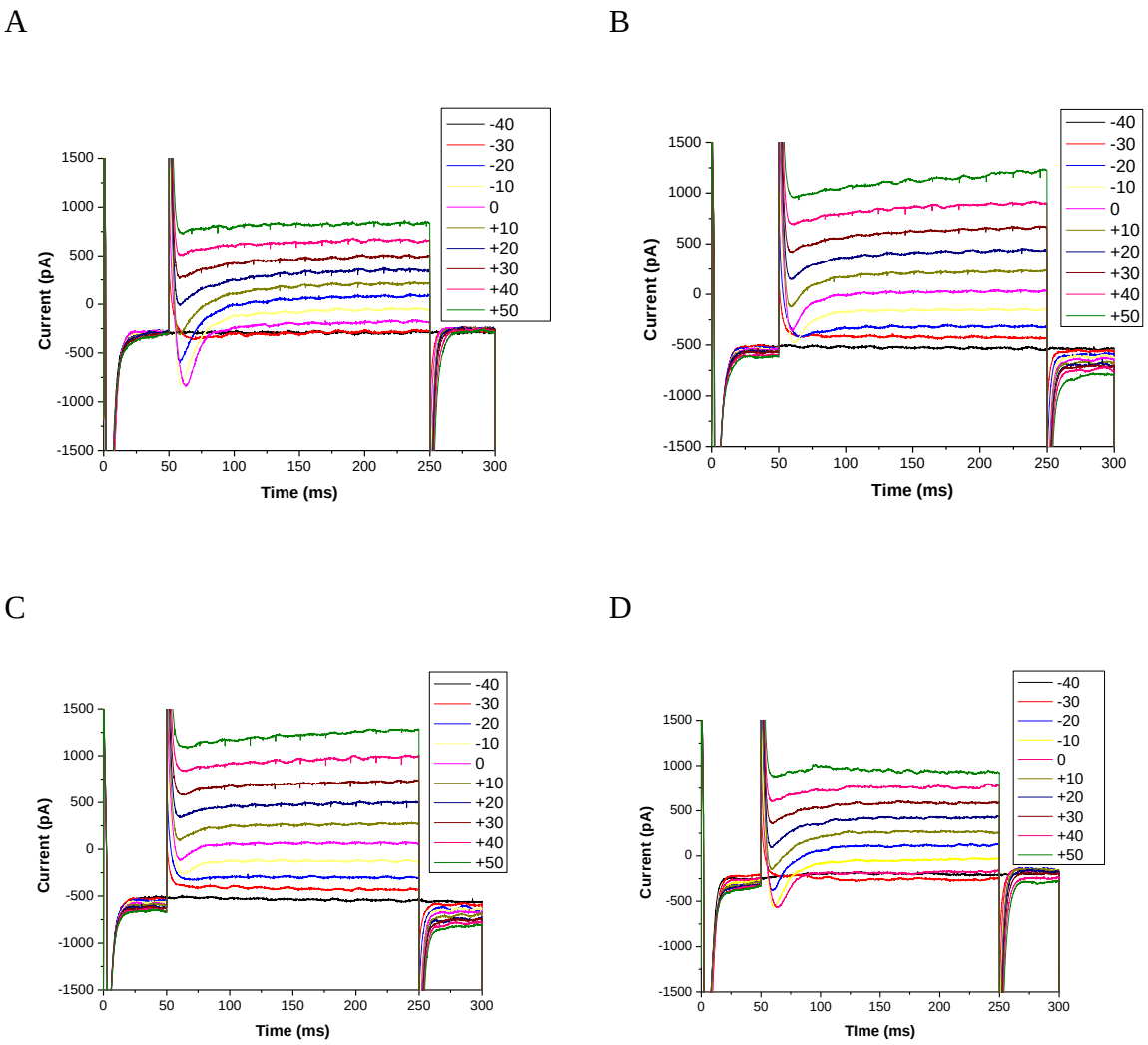


Figure 6.2: Typical recording of the L-type calcium channel (cell No. 1) current using multiple step depolarisation to a voltage range between -40 and +50 mV, A-control recording , B- treatment with esmolol (1 mM), C- treatment with esmolol (3 mM), D- calcium current after esmolol washout.

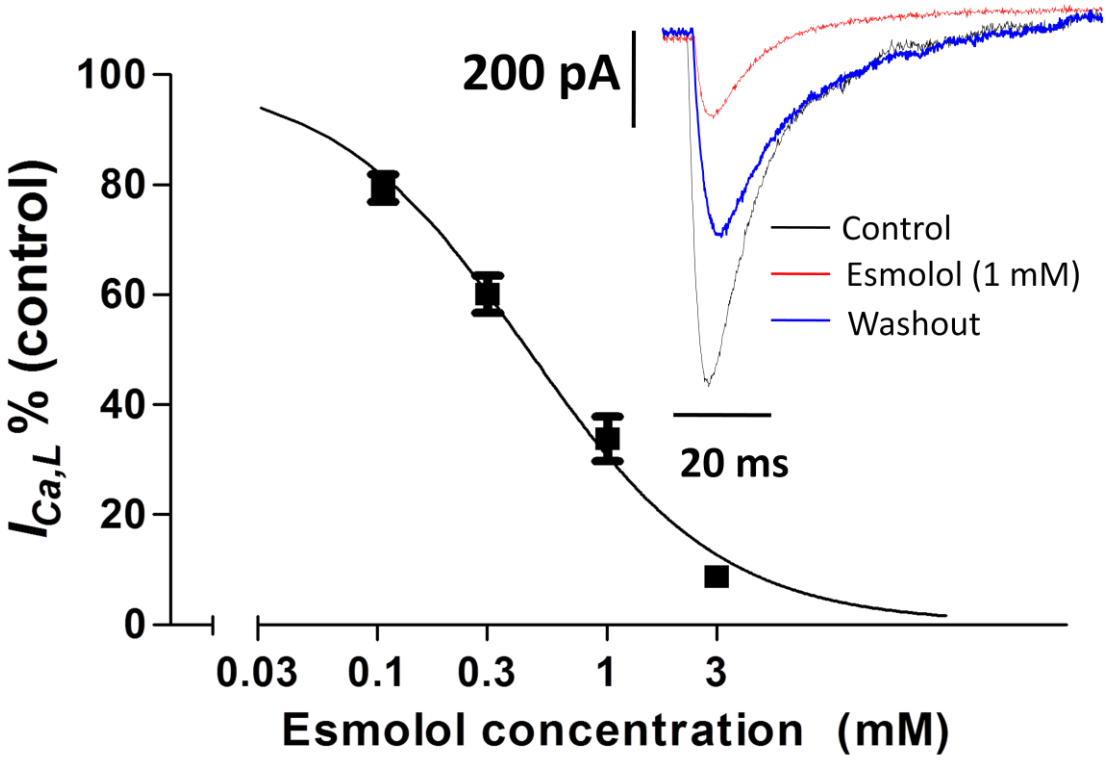


Figure 6.3: Concentration-response curve of esmolol inhibition on $I_{Ca,L}$ with example $I_{Ca,L}$ trace from cell No. 1 (inset) to compare the effect of esmolol (1 mM) with control and washout at -10 mV.

6.3.2 The voltage dependency of the effect of esmolol

Superfusing each myocyte with esmolol decreased the magnitude of $I_{Ca,L}$ without shifting the peak of the I-V curve (-10 mV) and at 3 mM esmolol, $I_{Ca,L}$ was nearly abolished. Figure 6.4 demonstrates that the inhibitory effect of esmolol on $I_{Ca,L}$ was equal at all voltages in all the myocytes included, indicating that $I_{Ca,L}$ block by esmolol was not voltage-dependent. After normalising this effect to the percentage of control current in order to offset to the size of the cell and the size of the currents at different voltages, esmolol indeed seems to have inhibited $I_{Ca,L}$ equally in all voltages (Figure 6.5) and therefore this inhibitory effect is clearly not voltage-dependent.

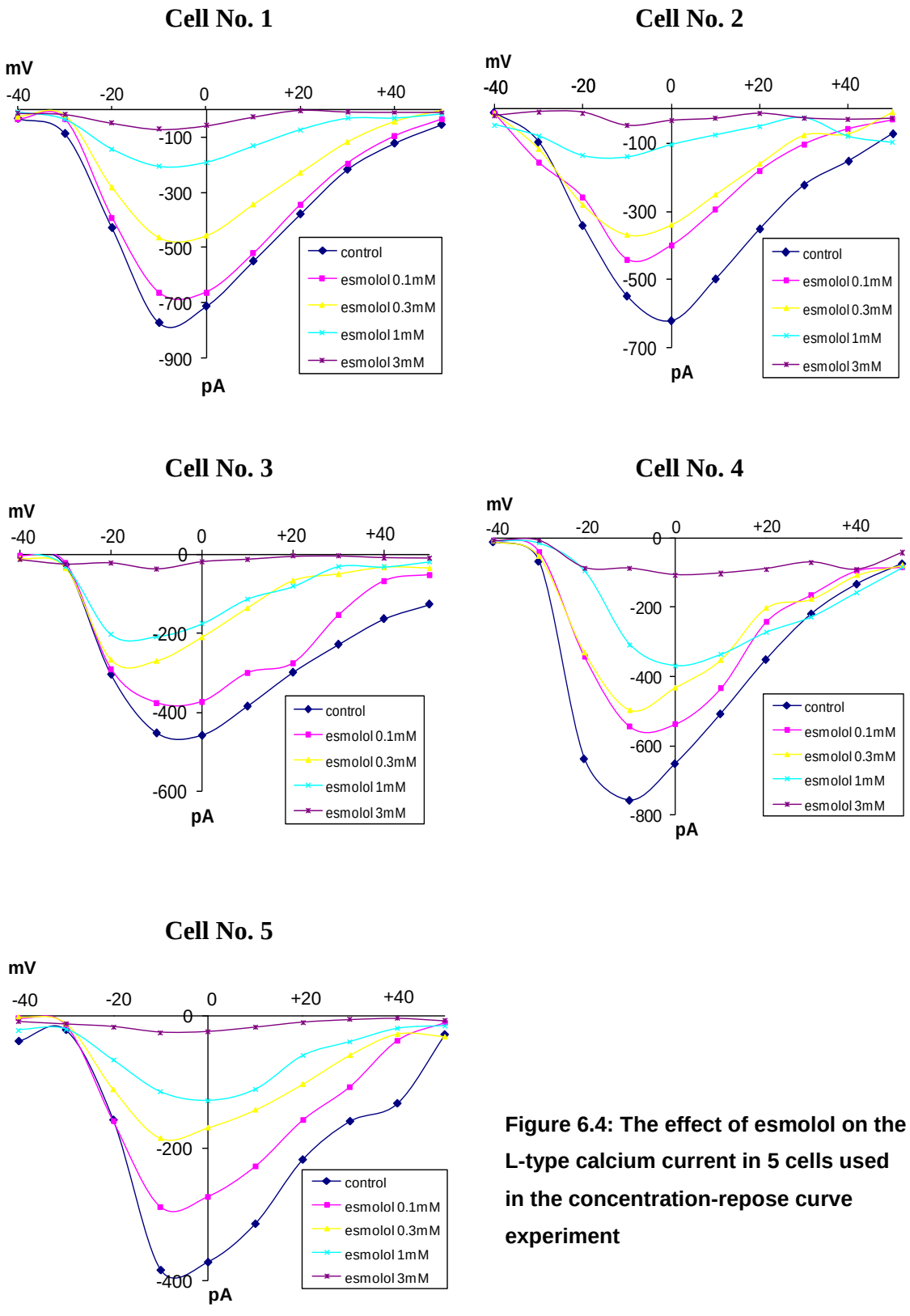


Figure 6.4: The effect of esmolol on the L-type calcium current in 5 cells used in the concentration-repose curve experiment

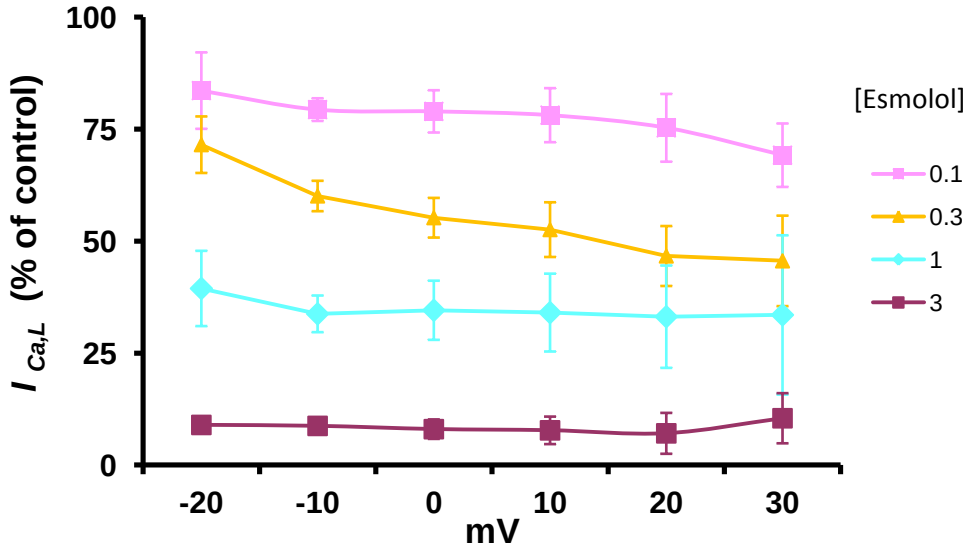


Figure 6.5 Voltage-independence of the inhibitory on $I_{Ca,L}$ at various concentrations of esmolol

6.4 **Discussion**

These studies have confirmed that inhibition of the Ca_{tr} by esmolol in isolated myocytes (Chapter 3) have been mainly by inhibition of the L-type calcium channels. This would have resulted in the negative inotropic effect in both the isolated myocytes, and in the Langendorff perfused rat heart of Bessho's study (Bessho and Chambers 2001). This novel effect is not widely accepted as it has always been assumed that any cardioplegic effect is achieved by β-blockade (Mehlhorn et al. 1999). It was only recently that a limited observational experiment (published shortly after starting this PhD project) showed a reduction in $I_{Ca,L}$ in pig and guinea-pig myocytes with 110 μM esmolol (Arlock et al. 2005). These studies were conducted after a profound negative inotropic effect of esmolol was observed while performing off-pump coronary bypass surgery on a patient. Arlock and colleagues using patch clamping techniques that were slightly different to our studies (they used the same method to inactivate the fast sodium current by stepping up to -40 mV initially and then they stepped straight away to 0 mV to activate and measure $I_{Ca,L.)}$ they found that esmolol at 110 μM reduced $I_{Ca,L}$ to 72% of control. Our findings in the rat myocytes were generally in line with those of Arlock's; we found that 100 μM esmolol resulted in a reduction of $I_{Ca,L}$ of 78 ± 4.7% at 0 mV.

At 1mM esmolol did not abolish $I_{Ca,L}$ totally; $I_{Ca,L}$ was inhibited to 34% of control in the isolated myocytes. Bessho and colleagues (Bessho and Chambers 2001) demonstrated that 1 mM esmolol induced complete ventricular arrest. Therefore, while the negative inotropic effect of esmolol can be sufficiently explained by the inhibition of $I_{Ca,L}$, the total arrest in the Langendorff hearts is still difficult to clearly understand. This poses the question of whether blocking the L-type calcium channels can entirely explain the arresting effect of esmolol in isolated hearts knowing that esmolol at 1 mM failed to totally inhibit the $I_{Ca,L}$ but yet was able to arrest the heart. In order to answer this question it is important to study the effect of esmolol on the fast sodium current, which is the first component in the action potential (as shown in Chapter One). Blocking the fast Na channel is one of the main mechanisms to induce cardioplegic arrest and this is how hyperkalaemic cardioplegia works. We intend, in the following chapter, to study the effect of esmolol on the fast Na- channels.

6.5 **Summary and Key Findings**

Esmolol was shown in this chapter to inhibit the L-type calcium channels using patch-clamp experiments. This inhibitory effect was dose dependent with an IC_{50} of 0.45 mM and was not voltage dependent

CHAPTER 7. RESULTS 5: THE EFFECT OF ESMOLOL ON THE FAST SODIUM CURRENTS

7.1 Introduction

Most non-selective β-blockers are known to have a Na-channel blocking effect at high doses and propranolol was proven to be one those agents (Ahrens-Nicklas et al. 2009). Despite being a selective β-blocker, esmolol was also shown to have such an effect at high doses. Deng and colleagues (Deng et al. 2006) demonstrated, using patch clamping studies that esmolol inhibits the fast sodium current (I_{Na}) in isolated rat ventricular myocytes. This mechanism could explain the observation in Section 3.3.1, where the threshold of myocyte excitation was increased with esmolol treatment. On the other hand, blocking the sodium channels can explain the negative inotropic effect of esmolol (Honerjager et al. 1986), possibly, by a reduction of intracellular [Na^+] and thereby, enhancing the extrusion of calcium from the cell via Na^+/Ca^{2+} exchanger, which would result in less releasable calcium stored in the SR. This effect requires a functioning SR and would be lost in the experiment in Section 4.3.1 where the SR was blocked. Esmolol however maintained its negative inotropic effect in the blocked SR, which does not support this effect entirely. Meanwhile, blocking the L-type calcium channels by esmolol (see previous chapter) can explain the negative inotropic effect of esmolol by reducing the intracellular calcium and therefore reducing the calcium-induced calcium-release (as explained in Section 1.5.5). However, it does not explain entirely the mechanism of arrest (as discussed in Section 6.4) and studying the effect of esmolol on the fast Na channels seems to be essential as the main trigger for the action potential in order to fully understand the arresting effect. Interestingly, Na channels are of great importance in cardioplegic arrest as the arrest induced by blocking the Na channels directly without shifting the membrane potential is expected to be polarised arrest, which could block the detrimental Na window current, which can be potentially beneficial compared to hyperkalaemic depolarised arrest as explained in detail previously (Sections 1.7.1.2).

The study by Deng and colleagues (Deng et al. 2006) demonstrating that esmolol inhibits fast sodium channels were conducted in isolated rat ventricular myocytes using patch clamp techniques; starting from a holding potential of -120 mV to achieve maximum activation of the channels, an inhibitory effect with IC_{50} of around 75 µM was demonstrated. In this chapter, we study the difference between I_{Na} generated from a holding potential of -120 mV and a more physiological resting membrane potential of -90 mV. We also looked at the concentration-response relationship and the voltage dependence of esmolol inhibition to I_{Na} from holding potentials of -90 mV.

7.2 **Methods**

Measuring I_{Na} current was slightly harder to obtain than $I_{Ca,L}$ due to the easy distruption of the seal and the detrimental effect of the slightest leak in the seal on the cell and the current. I was assisted during these experiments by Dr. McLatchie who has long experience in patch clamping. These studies were conducted according to the method described in detail earlier in Sections 2.2.4 and 2.4. In brief, rat ventricular myocytes isolated according to the method mentioned in Section 2.3.1. A drop of the myocyte suspension was mounted in the perfusion chamber and the myocytes were allowed to settle on the glass bottom of the chamber for a few minutes. The myocytes were subsequently superfused with Tyrode solution. The electrodes were made from heat pulled glass pipette and filled with the Na^+ patch clamping internal solution (Figure 2.8). The electrode resistance was measured with the tip immersed in the bath solution. The pipette of resistance between 1.5 and 3 MΩ were used to ensure appropriate tip diameter. A hydraulic micromanipulator (MO-102, Narishige) was used to position the suction glass electrodes. A tight seal is formed between the tip of the pipette and the surface of the myocyte using the micromanipulators under x40 microscope objective l. Seal resistance of greater than 1 GΩ was achieved by applying negative pressure (30-60 cm/H_2O). The patch membrane was then ruptured by a brief period of stronger suction. If an access resistance less than 6 MΩ was achieved, the Tyrode solution was switched to the external solution in the I_{Na} experiments (Figure 2.8). The external solution was then superfused at 23^{o}C only because it was not possible to maintain consistent seals for the length of the experiment at a more physiological temperature of 34^{o}C. Currents were sampled at 10 kHz and filtered at 5 kHz. pCLAMP 10 software (Axon Instruments) was

used for the acquisition and analysis of currents. After a short period of stability the I_{Na} was initially measured from a holding potential of -120 mV and elicited by multiple steps to membrane potentials between -80m to +10mV. Then, this measurement was repeated by stepping from a more physiological resting membrane potential (-90 mV) using the same steps to establish the I/V relationship for both holding potentials. An attempt to measure I_{Na} by stepping from a holding membrane potential of -80mV did not generate a measurable current. The I_{Na} was the negative deflection immediately after stepping up and it is faster than the $I_{Ca,L}$ measured in the previous chapter as it was resolved within 25 ms with a peak around 10 ms (Figure 7.1). The amplitude of this current was calculated by measuring the difference between the peak of the curve and the flat line 150 ms. later. For some measurements, there was noise in the flat line at around 50-60Hz, which was in the shape of regular wave form with an amplitude of no more than 50 pA; in order to avoid any error in determining the value of the flat line, the curve was averaged over a period of 20 ms and the average value was subtracted from the peak. This was normalised to the control when stated or normalised to the capacitance of the measured myocyte as the size of the current depends on the size of the cell and therefore its capacitance. The concentration-response relationship and rate dependency were studied using the holding membrane potential of -90mV. I_{Na} was activated using a 10 step pulse train from -90mV to -50mV.

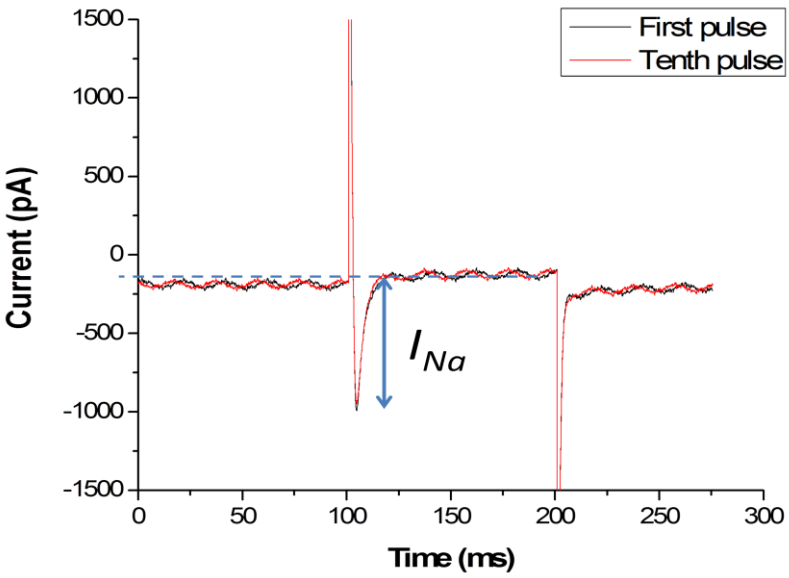


Figure 7.1 Example trace of the I_{Na} and the method of calculation

7.3 **Results**

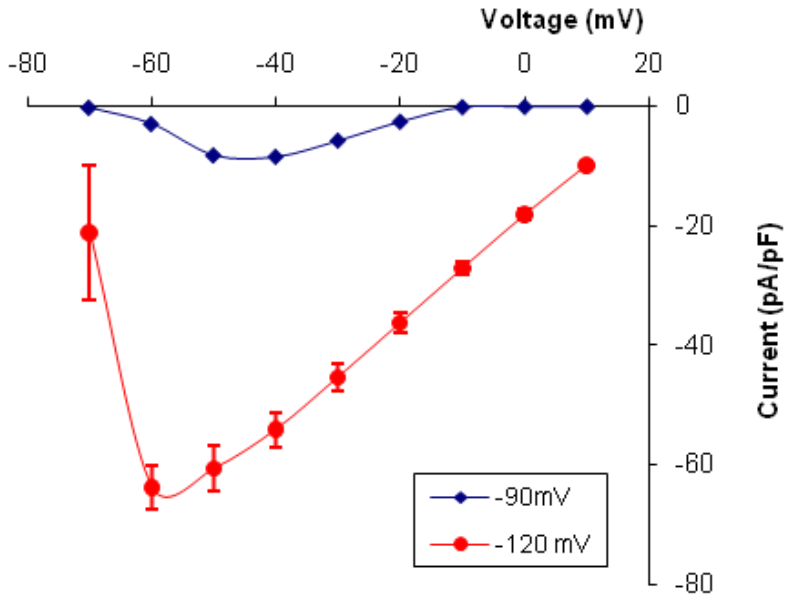
7.3.1 The I/V relationship study

In this chapter, our aim was to measure the blocking effect of esmolol on the fast Na-channels from physiological holding potentials, and compare theses results with the findings of Deng and colleagues (Deng et al. 2006) in, which a hyperpolarising holding potential of -120 mV was used. The I/V relationship from stepping from either -90mV or -120mV was studied using the protocol mentioned above. Four cells (n=4) were adequate to demonstrate the I/V relationship variation between the holding potentials -90 and -120 mV. The results of individual cells are outlined in detail in Table 7.1. The I_{Na} currents generated from the holding voltage of -120 mV were, almost 10 times larger than the ones generated from the holding voltage of -90 mV after normalising to cell capacitance (Figure 7.2). We also found that the peak I_{Na} activation was at -60 mV for the hyperpolarisation holding voltages, whereas this peak of I_{Na} activation had shifted slightly towards depolarisation of -40 to -50 mV in the activation step from the holding potential of -90 mV (Figure 7.2).

Activation voltage (mV)	I_{Na} Current (pA/pF)			
	Cell A	Cell B	Cell C	Cell D
Stepping from -90 mV				
-70	0.37	0.43	0.22	0.18
-60	4.54	2.58	2.69	2.10
-50	10.40	5.29	9.42	7.49
-40	9.87	4.66	10.10	8.89
-30	6.89	2.07	7.31	6.57
-20	3.31	0.11	3.53	3.45
-10	0.22	0.10	0.18	0.27
0	0.10	0.12	0.25	0.16
10	0.04	0.08	0.12	0.22
Stepping from -120 mV				
-70	4.69	25.63	51.11	3.23
-60	58.84	74.66	59.23	62.80
-50	57.96	71.01	53.38	60.35
-40	51.98	62.21	48.69	53.60
-30	44.33	51.59	40.21	45.29
-20	35.83	40.79	32.19	36.22
-10	27.23	29.84	24.30	27.25
0	18.25	20.12	16.08	18.29
10	10.04	10.62	8.27	10.55

Table 7.1 I/V relationship data of individual myocytes demonstrating the difference between holding potentials of -90 mV and -120mV

A



B

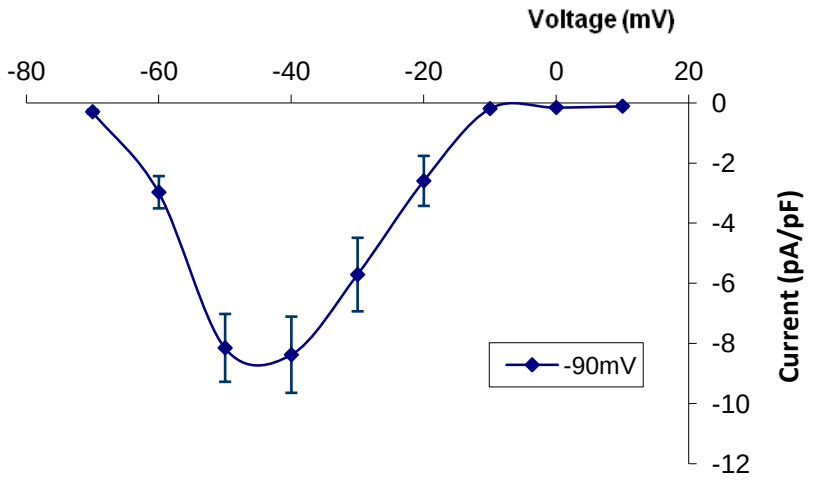
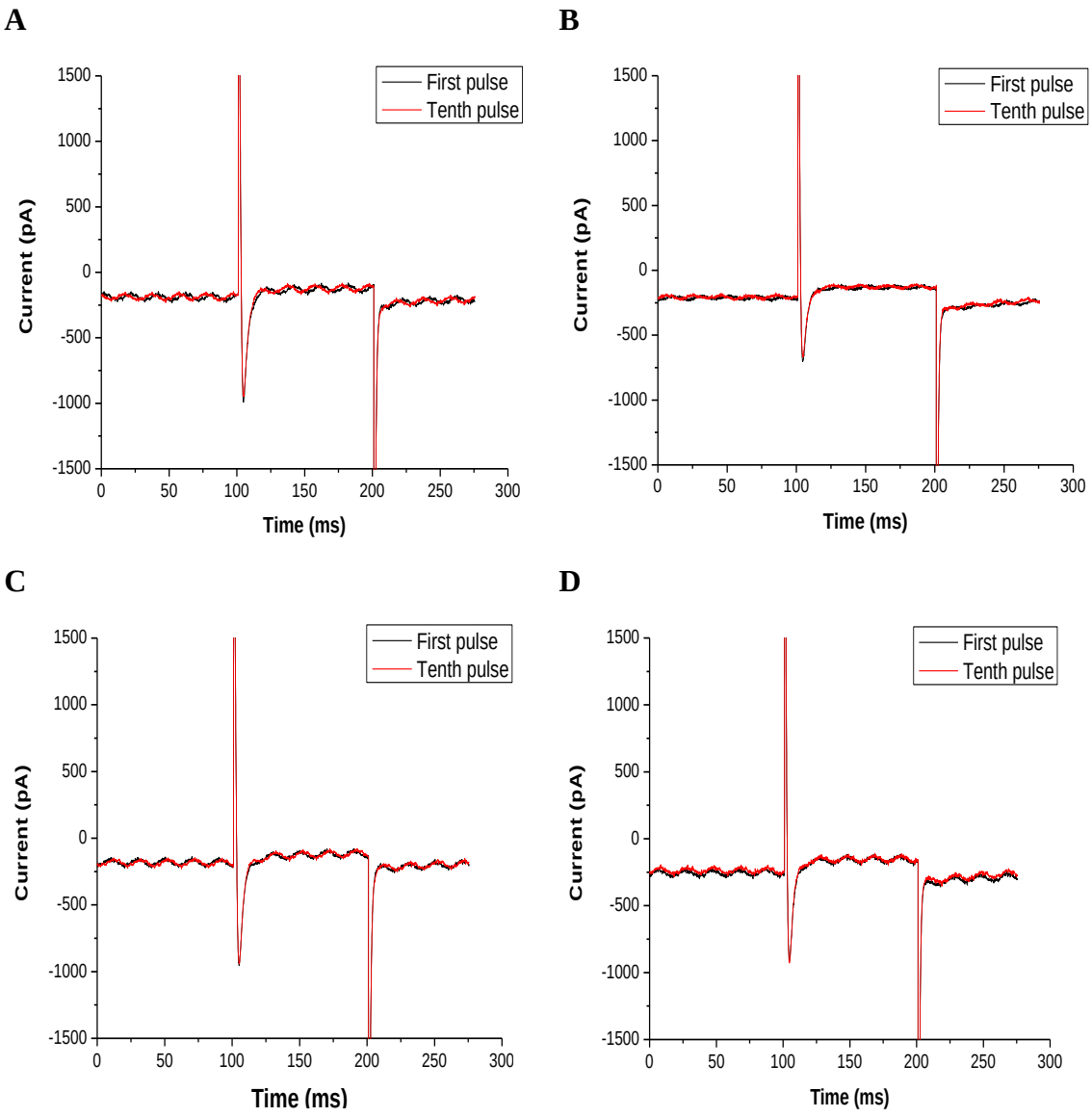


Figure 7.2: I/V relationship of I_{Na} , A- comparison between stepping from holding potential of -120 mV or-90 mV. ; B- Large scale graph of -90 mV. (*n*=4).

7.3.2 The dose-response curve and rate dependency effect of esmolol on I_{Na}

As explained earlier, after stabilisation, baseline I_{Na} was measured from holding potential of -90 mV stepping to -50 using a 10-pulse train. The myocytes were superfused with increasing concentrations of esmolol (from 0.01-1.0 mM). We found that, while 0.1 mM esmolol inhibited I_{Na} by around 10-20%, the next dose of 0.3 mM had inhibition that was close to 90%. To study this part of the steep curve, we included another concentration of esmolol at 0.2 mM. We also found that lower concentrations (0.01 and 0.03 mM), in some occasions, has slightly increased I_{Na} (as shown in Figure 7.3, which demonstrates a typical example of I_{Na} in a cell superfused with the increasing dose of esmolol and then a washout current). It was difficult to be certain that this effect was indeed due to the small esmolol dose or whether it was due to variation in the measurements. At 1 mM/L, esmolol completely abolished I_{Na}, which was reversed partially after 3 min of wash out of the drug. There was no difference between the first and the tenth pulse at any of the measurements, which excludes any rate dependency in the inhibitory effect of esmolol on I_{Na}. The I_{Na} value was normalised to baseline measurement and a dose response curve was constructed for the first and the tenth pulse (Figure 7.4). This figure also shows a typical example of the effect of esmolol (0.2 mM) on I_{Na}. The IC_{50} values for the first and tenth pulses were not different (0.169 ±0.025 and 0.162 ± 0.024 mM, respectively; Figure 7.4). Thus, we observed no rate-dependency of the inhibition of esmolol on I_{Na} .



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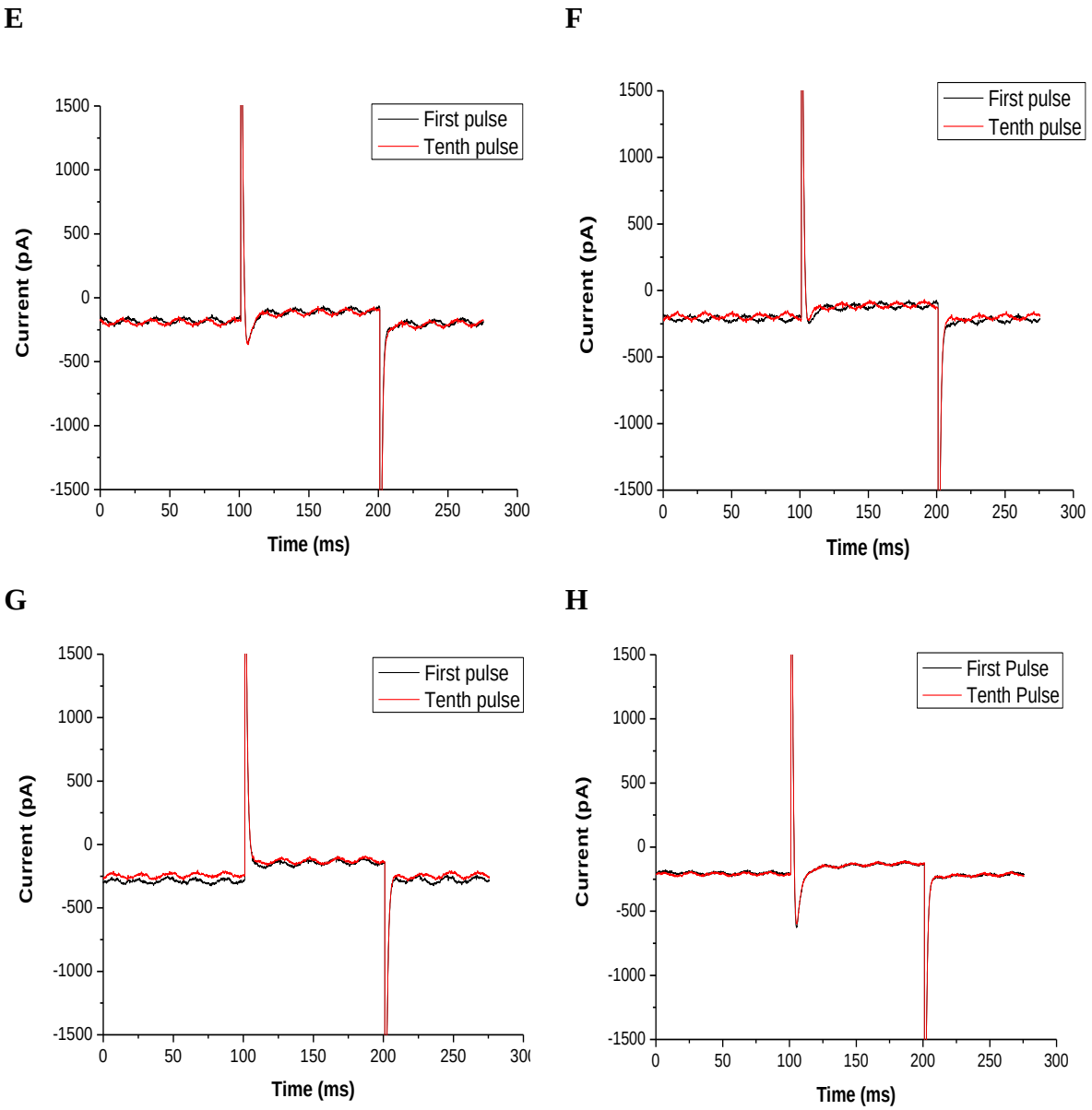

Figure 7.3: Typical recording of I_{Na} channel (cell No. 1) using a train of 10 pulses with step depolarisation from -90 mV to -50 mV. A-control recording , B- treatment with esmolol (0.01 mM), C- esmolol (0.03 mM), D- esmolol (0.1 mM). E- esmolol (0.2 mM), F- esmolol (0.3 mM), G- esmolol (1 mM), D- Washout for 3 minutes

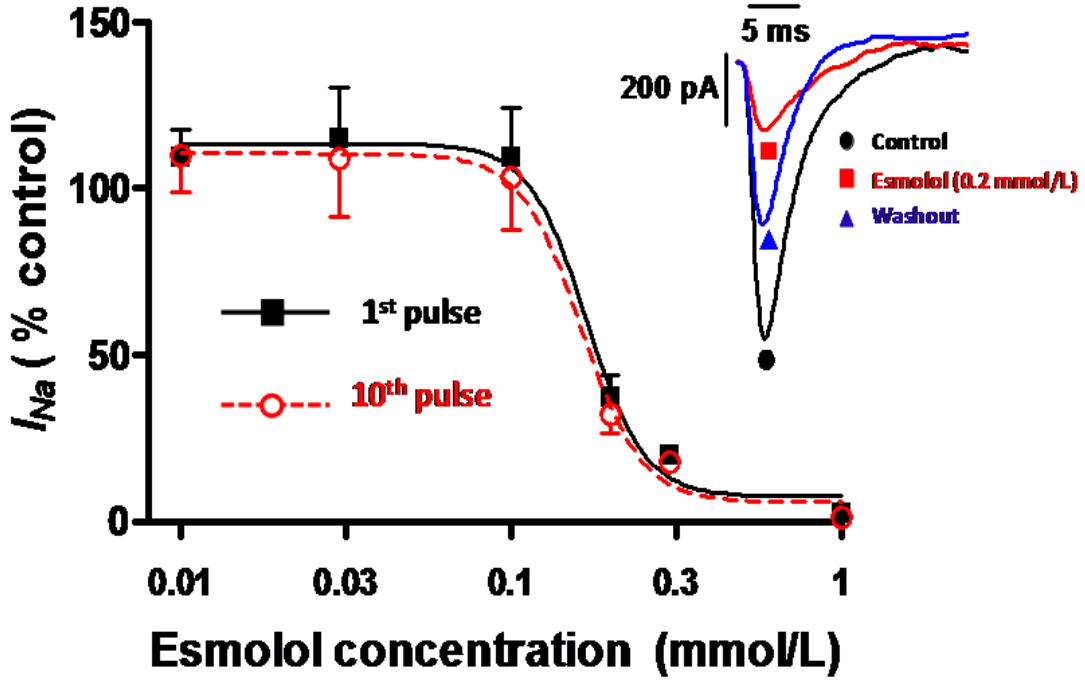

Figure 7.4: Concentration-response curve of esmolol inhibition on I_{Na} with example trace I_{Na} to compare the effect of esmolol (0.2 mM) with control and washout at -50 mV (inset)

7.4 Discussion

These studies demonstrated that esmolol inhibits I_{Na} at lower concentrations than those for the inhibition of $I_{Ca,L}$. (with an IC_{50} ~ 0.16 vs. 0.45 mM respectively). Interestingly, esmolol at 0.3 mM almost abolished I_{Na}. However, we know from our previous studies (Bessho and Chambers 2001) that this concentration did not achieve full arrest in the Langendorff perfused rat hearts that a higher concentration was required to abolish $I_{Ca,L}$ as shown in the studies in Chapter 6. This suggests that total inhibition of I_{Na} is not sufficient to induce arrest in these studies. Sodium channel blockers are known to have a negative inotropic effect (Honerjager et al. 1986), possibly because they tend to reduce intracellular [Na^+] and thereby enhance the extrusion of Ca^{2+} from the cell due to the increasing the gradient of Na^+ across the membrane, which drives the Na^+/Ca^{2+}

exchanger; this would result in less releasable Ca^{2+} stored in the SR. It is, therefore, possible that an effect of esmolol on the fast Na^{+} channel contributed to the negative inotropy in submillimolar doses of esmolol reported in the isolated myocytes studies (Chapter 3) and observed by Bessho (Bessho and Chambers 2001) in the rat hearts, as well as during cardiac surgery (Arlock et al. 2005). However, it is unlikely that the inhibition of I_{Na} is the predominant mechanism for negative inotropy and arrest because the negative inotropic effect of esmolol was not affected by SR inhibition (Chapter 4CHAPTER 4), and the inhibitory effect of selective I_{Na} blockers on contraction is smaller than on I_{Na} (as measured by upstroke velocity of the action potential) (Honerjager et al.). We therefore conclude that the inhibition of I_{Na} is likely to be a contributing factor but that the major mechanism of action for the negative inotropic effect of esmolol and arrest is the inhibition of $I_{Ca,L}$.

7.5 **Summary and Key Findings**

The effect of esmolol on the fast sodium current was investigated in this chapter using patch-clamp experiments. We demonstrated that esmolol inhibited the fast Na current at -90 mV in a dose dependent manner. The inhibition was found to be comparable in the first and tenth pulse, which concludes that it was not rate dependent inhibition.

CHAPTER 8. RESULTS 6: ESMOLOL EFFECT IN THE ISOLATED RAT HEART

8.1 Introduction

The previous studies have demonstrated that esmolol directly inhibits contraction of the ventricular myocytes. However, cardiac arrest in the whole heart could be due to either direct inhibition of contraction at the level of the myocytes or inhibition of action potential at the level of the conduction system. Work from our lab by Bessho and colleagues (Bessho and Chambers 2001) has previously demonstrated that whole heart perfusion with esmolol only (without any ischaemia) resulted in a dose-dependent negative inotropic effect by inhibiting LVDP at lower concentrations (0.3 mM). Further increase in esmolol concentration (1 mM) induced full arrest at the level of the ventricle, whereas atrial arrest required a higher (3 mM) concentration. (Figure 1.13). The dose dependent negative inotropic effect on the ventricle demonstrated in this experiment can be explained by the previous findings in Chapters 3 to 7 in the isolated myocytes in particular the L-type calcium channel blocking effect in Chapter 6. However, the atrio-ventricular dissociation that occurred with esmolol at 1 mM suggests an effect on the conduction system; in particular, the AV node. Sodium channel blockers have been shown to partially inhibit the pacemaker cells in the AV node (Marger et al. 2011). Meanwhile, L-type calcium channel blockers are well known to induce complete heart block at the level of the AV node (Regan et al. 2005; Marger et al. 2011). It is possible, therefore, that esmolol did indeed induce arrest by blocking the AV node through the sodium and L-type calcium channel blocking effect. The other possible mechanism can be explained by the poor coronary perfusion to the right atrium in the isolated rat heart preparation as the main supply to the right atrium from the SA node artery (Yamazaki et al. 2010) can get damaged easily while harvesting the heart. This could result in reduction of the bioavailability of esmolol in the atrial tissue and the SA node, which explains the need for a higher concentration to induce atrial arrest. The aim of this chapter is to investigate the mechanism of arrest in the whole heart and to

determine whether, the arrest at the level of the ventricle in Bessho's work (Bessho and Chambers 2001) (Figure 1.13) is either due to direct inhibition of myocytes contraction or failure of the pacemaker/ conductive system of the AV node. Furthermore, we aim to explain the lack of atrial arrest by esmolol (1 mM) in these experiments, which may be explained by the reduction in atrial superfusion.

8.2 **Methods**

The isolated of the rat hearts and Langendorff perfusion methodology are explained in detail in Sections 2.2.7 and 2.5. In brief, after rapid harvesting of the heart and immersion in ice-cold perfusate, it was canulated and perfused with KHB solution within 30 to 60 seconds to minimise the length of ischaemia. After removing the left atrium, a ventricular balloon was inserted into the left ventricle. The balloon was filled with water and the pressure was calibrated to maintain intra-ventricular diastolic pressure of 4-8 mmHg. The heart is kept in a water-jacketed glass reservoir covered with paraffin film 'parafilm' to maintain the temperature, and was then perfused with KHB at 37^{o}C. For these experiments, an additional cannula was placed over the right atrium to superfuse the atrial tissue in order to overcome any potential low perfusion to the atrium as explained above. We also inserted a pacing lead on the ventricle to allow pacing the heart in order to distinguish between the arrest induced by complete heart block or by direct inhibition to the myocardium. A unipolar electrocardiogram (ECG) was obtained using an electrode inserted into LV (Figure 8.1). The heart was then equilibrated for a 20 minutes stabilisation period. The values were, again, presented as percentage of control, measured at the end of the stabilisation period. The sigmoid curves were fitted using GraphPad Prism software to the following equation: $Y= y_{min} + (y_{max}-y_{min})/(1+(IC_{50}/X)^{-n})$ where X is the drug concentration, IC_{50} is the concentration for 50% of the maximal inhibition, and n is the Hill coefficient. We randomised 5 hearts per dose of esmolol and examined doses of 0.03, 0.1, 0.3, 1 and 3 mM. In order to establish the mechanism of arrest in the whole heart we designed an experimental protocol, which aimed to investigate the mode of action in each part of the rat heart by studying the effects of esmolol with and without atrial superfusion or ventricular pacing.

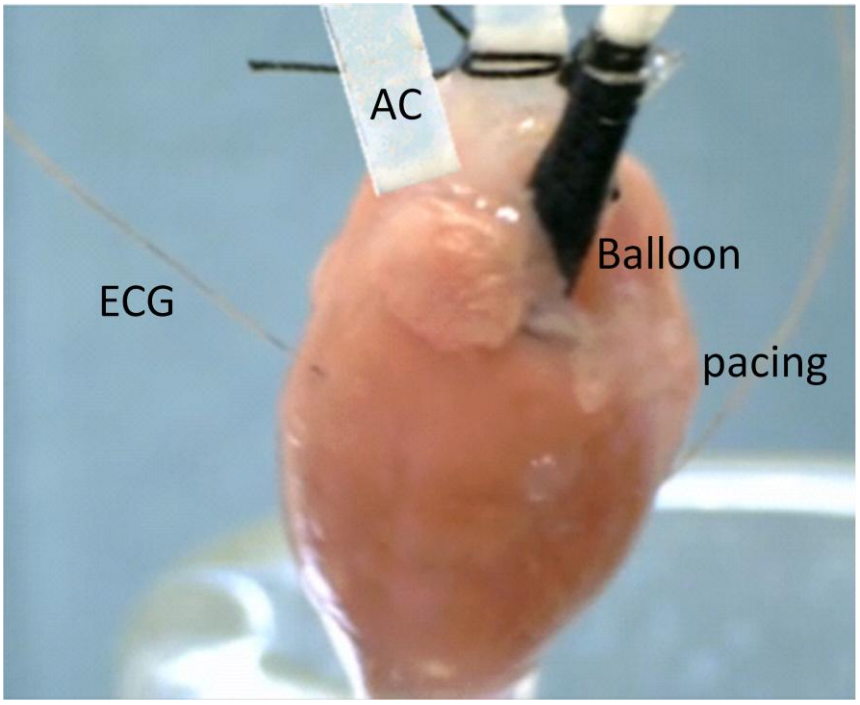

Figure 8.1 A photograph of a Langendorff perfused heart with a diagram of the atrial superfusion cannula placed above the right atrium (AC). An ECG lead and pacing leads and the site of the ventricular balloon insertion.

LVDP, ventricular heart rate and left ventricular diastolic pressure were measured by averaging the ventricular balloon trace over a period of 30 seconds. Atrial heart rate was also averaged over 30 seconds from the ECG trace. Coronary flow measured from the perfusate collected in the heart chamber over 3 minutes. In the atrial superfusion phase, this volume represents a mixture of the atrial superfusate and the coronary flow. Therefore, the coronary flow could not be measured correctly using this method and for simplicity purposes, we did not measure coronary flow during this phase. The experimental perfusion protocol is shown in Figure 8.2 consisted of: 20 min stabilisation period with Langendorff retrograde perfusion alone without atrial superfusion (control measurement) and then a 3- minute period with additional atrial superfusionm a return to coronary perfusion alone (3 min); 3 min LV pacing (360 b.p.m), then 3 minutes of no pacing. The KHB alone was then replaced with KHB containing esmolol (one randomised concentration per heart) for 5 minutes and the entire perfusion sequence was repeated with atrial superfusion phase and ventricular pacing phase (Figure 8.2). After that the hearts were allowed to recover for 20 minutes and LVDP was measured again.

Figure 8.2 A diagrammatic representation of the perfusion protocol, (blue bars represents KHB perfusion, brown bars represents esmolol perfusion, the green bars represents the sampling time). KHB: Krebs Henseleit Buffer, At: atrial, V:ventricular, Esmolol: KHB+ esmolol.

8.3 Results

8.3.1 Validating the protocol during KHB perfusion

We initially needed to establish that the protocol preceding the esmolol perfusion did not influence LVDP or heart rate. This was important in order to ensure the protocol did not alter any esmolol responses and to determine the control point during the pre-esmolol perfusion. LVDP and heart rate remained stable with no significant changes throughout the KHB perfusion period with the exception of heart rate during the pacing period at 360 bpm (Figure 8.3). Similarly, the coronary flow rate remained stable throughout this stage with, an average of 13 ml/min.

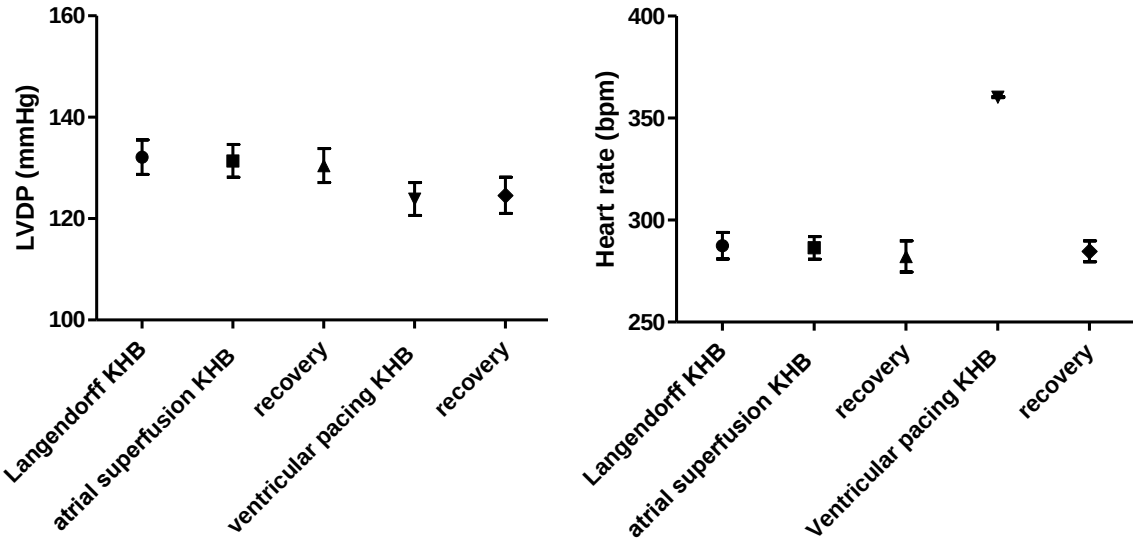

Figure 8.3 The change in LVDP (left) and heart rate (right) during KHB perfusion for all the hearts (*n*=25)

8.3.2 The dose-response relationship of esmolol with or without atrial superfusion

The heart rate during KHB perfusion did not change during atrial superfusion; heart rate was 289 ± 13 bpm during KHB perfusion alone *vs.* 290 ± 11 bpm during KHB with atrial superfusion. Langendorff perfusion with 1 mM esmolol drop the atrial rate; in contrast, atrial superfusion resulted in 2 hearts achieving full atrial arrest electrically and a substantial decrease in the atrial rate of the other 3 hearts to giving a mean heart rate of 235 ± 13 bpm (Figure 8.4).

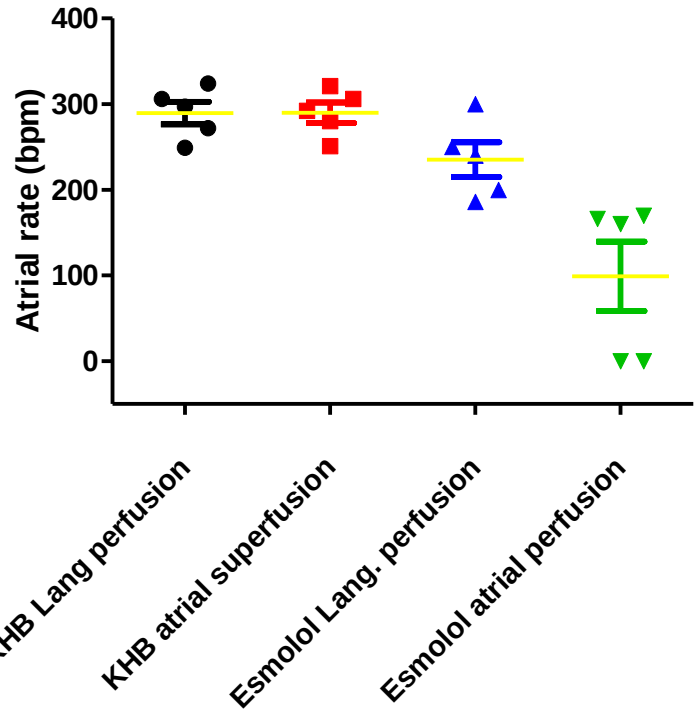

Figure 8.4 Atrial rate variation in the esmolol (1 mM) hearts (*n=5*) demonstrating the effect of esmolol on atrial rate with Langendorff perfusion only and a more profound effect with atrial superfusion. (yellow bars are the Mean, error bars are SEM)

While 0.03 mM of esmolol had negligible effect on heart rate with and without atrial superfusion it inhibited LVDP significantly to 78±3% of control. Higher sub arresting doses (0.1 and 0.3mM) of esmolol have reduced heart rate to 86±2% and 73±6% of

control respectively in normal perfusion and 85±3% and 68±5% of control respectively with atrial superfusion. The inhibitory effect of these doses on LVDP was more profound to 51±4% and 20±6% of control respectively. 1 and 3 mM esmolol induced arrest similar to findings by Bessho and colleagues (Bessho and Chambers 2001)

8.3.3 The dose-response curve of esmolol in paced *vs*. unpaced hearts with standard perfusion

This study was conducted to establish whether the ventricular arrest induced by esmolol is caused by an inhibition of the conduction system, in particular the A-V node. In order to achieve this, we constructed the dose-response curve of esmolol on LVDP in the initial Langendorff perfusion phase normalised to the LVDP measured at stability (control). This was then compared to the dose-response curve of esmolol in the paced phase normalised to the same period. As expected, heart rate was different between the two phases; esmolol concentrations below an arresting threshold dose did not slow heart rate during the paced phase, whereas these doses reduced heart rate during the spontaneously beating phase (Section 8.3.2). This variation in heart rate, however, did not alter the inotropic response and the inhibitory effect on LVDP in the paced phase comparative to the unpaced phase. By constructing the average sigmoid curve of this dose-response relationship of esmolol on LVDP in the paced versus the unpaced hearts, the IC_{50} values were 0.109 ± 0.014 mM and 0.097 ± 0.013 mM, respectively with the Hill coefficient values being 1.3±0.19 and 1.3±0.19, respectively (Figure 8.5). At 1 mM esmolol, pacing the heart failed to generate any contraction, which confirms that esmolol inhibited the contraction directly at the level of the myocardium rather that the conduction system.

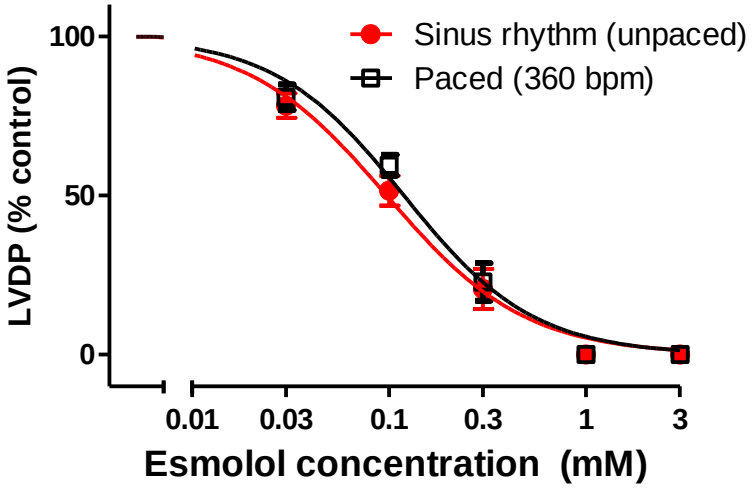

Figure 8.5 Concentration–response curves for esmolol in Langendorff-perfused hearts demonstrating the effect on LVDP in ventricular paced (360 b.p.m.) *vs.* unpaced (280±50 b.p.m.) hearts

8.3.4 Esmolol dose-dependent recovery studies

The esmolol concentrations used to induce heart arrest are very high compared with the normal plasma concentrations of ~ 10-100 µM (Adamson et al. 2006) when esmolol is used clinically as a β-blocking agent due to its hypotensive ability. Therefore, it was important to ensure that, at these high concentrations, esmolol does not have a long lasting-detrimental effect on the heart. Equally, we needed to establish the optimal dose used to induce effective arrest, and at the same time ensuring that this effect is reversible as soon as possible after esmolol is washed out. In order to do this, hearts were perfused for an additional 15 minutes with KHB alone after the protocol shown in Figure 8.2, LVDP, and heart rate were measured at 0, 1, 3, 5, 10, and 15 minutes and recovery is shown in Figure 8.6 and Figure 8.7. The reversibility of the negative inotropic effect of esmolol is dose-dependent, with 0.3 mM appearing to demonstrate a rapid reversibility very instant reversibility with an improvement in LVDP compared to control. A concentration of 1 mM esmolol also showed complete recovery of LVDP, but it was significantly slower with partial recovery in heart rate.

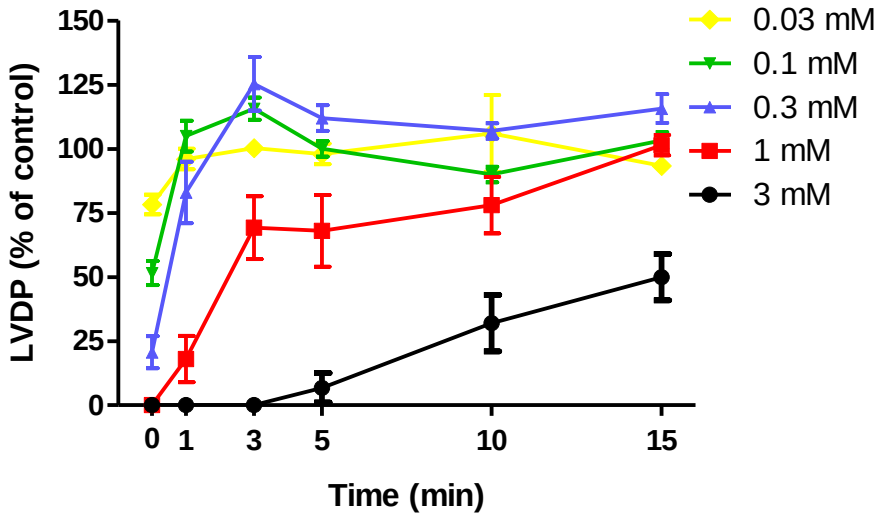

Figure 8.6 Washout of the effect of esmolol on LVDP. Time zero represents LVDP at the end of the perfusion with esmolol, before washout with KHB. Data are normalised to the values measured during control superfusion with KHB alone

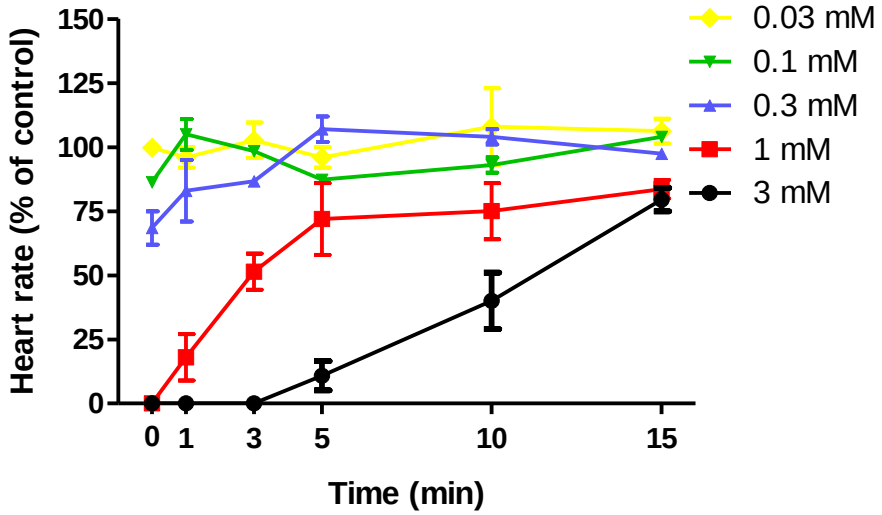

Figure 8.7 Washout of the effect of esmolol on heart rate. Data are normalised to the values measured during control superfusion with KHB alone.

8.4 Discussion

The studies described in previous chapters demonstrated that esmolol exhibited negative inotropic effects at the level of the myocytes. In addition, Bessho and colleagues

(Bessho and Chambers 2001) have showed that 1mM esmolol resulted in an atrio-ventricular dissociation, which may have been as a direct block of the conduction system, in particular the AV node. This mechanism is very feasible, as sodium and calcium channel blockers have been shown to have inhibitory effect on the AV node (Regan et al. 2005; Marger et al. 2011). It was important to confirm that arrest in the whole heart was not limited solely to inhibition of conduction without direct inhibition of myocardial contraction, in order to evaluate the suitability of esmolol arrest in cardiac surgery. It is not feasible to rely on cardioplegic potential for an agent, which has residual excitability of the myocardium for many practical reasons; during surgery, it is possible that the heart becomes excitable by directly handling the ventricles during surgery, which could result in contraction while working on the heart. This could be potentially dangerous during critical surgical manoeuvres or even by exposing the heart to contraction during ischaemia as it increases energy consumptions. Another clinical scenario where such arrest is not feasible during surgery is in patients with an internal pacemaker, which might result in failure to induce arrest altogether. The findings Section (8.3.2) reproduced those of Bessho and colleagues' (Bessho and Chambers 2001) demonstrating the negative inotropic effect of esmolol on the ventricle by reducing the LVDP significantly and inducing full ventricular arrest at 1 mM. We also reproduced the lack of atrial arrest electrically using 1 mM in the standard Langendorff perfusion method. Moreover, we demonstrated that by superfusing the atrium with esmolol we managed to increase the arresting effect and the negative chronotropic effect of esmolol on the atrium. After normalising the LVDP and heart rate to the control measurements following the stabilisation period we constructed a dose response relationship in the same manner as in Bessho's study (Bessho and Chambers 2001), which is depicted in Figure 1.13 in the First chapter and is shown again in Figure 8.8 alongside the data from this study. The effect of atrial superfusion on atrial rate was found to be statistically significant in the 1 mM group using paired t-test ($P<0.05$). At 0.3 mM atrial superfusion further decrease the heart rate including the ventricular rate without inducing ventricular arrest, which explains the difference between the average in atrial rate during atrial superfusion, which is in fact the heart rate and ventricular rate during Langendorff perfusion alone in this dose group in Figure 8.8.

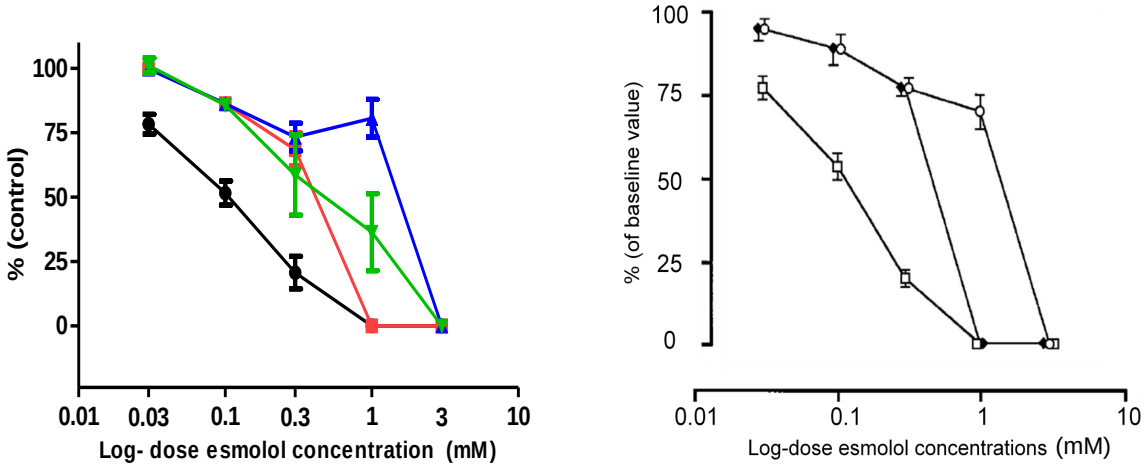

Figure 8.8 Concentration-response curves for the effect of esmolol comparing our findings (left panel) with Bessho's published results (right panel). Left showing effect on LVDP (●), ventricular rate (■), atrial rate (▲), and the atrial rate during additional atrial superfusion (▼). Right panel is duplicate of Figure 1.13 taken from Bessho and colleagues (Bessho and Chambers 2001) showing LVDP (□) ventricular rate (○) and atrial rate (◆).

This study clearly does not exclude the inhibitory effect of esmolol on the conduction system in general and the AV node in particular. It does, however, confirm that esmolol arrest in the whole heart is not caused or limited by this mechanism and that the direct inhibitory effect on the isolated myocytes shown in earlier chapters is the main arresting mechanism. It is, therefore, appropriate to use as a cardioplegic arrest in heart surgery for the reasons mentioned above.

The washout studies later have confirmed that the reversibility of esmolol's effect on LVDP and heart rate is dose dependent; while 1 mM offered robust and effective arrest, the recovery was partial in heart rate and slow in LVDP recovery despite being complete after 15 minutes; it was slower than in 0.3 mM. On the other hand 0.3 mM arrest was not consistent. This has suggested the question whether inducing arrest was the main important factor in determining the reversibility of esmolol in the 0.3 mM group. By performing subgroup analysis onto 0.3 mM treated hearts we found that the 2 arrested heart average LVDP recovery was very close to the three unarrested hearts. This excludes the possibility that recovery from esmolol treatment at these doses is dependent on whether arrest has been induced or not.

In summary, this study demonstrated that esmolol can offer a practical cardioplegic arrest by direct inhibition of the myocardium, which makes it appropriate to be used in cardiac surgery. There is a concentration window where esmolol can provide effective arrest but can still show efficient and rapid reversibility during reperfusion and the window appears to be between 0.3 mM and 1 mM in the rat hearts with this crystalloid preparation. Further studies are required to establish whether these data will translate into larger animal hearts and in human studies.

8.5 **Summary and Key Findings**

The effect of esmolol was studied in the Langendorff perfused rat hearts in order to establish whether the arrest in the heart was due to the inhibition of the conduction system only or whether esmolol's inhibitory effect was at the level of the myocardium. We demonstrated that esmolol induced ventricular arrest at 1mM. Meanwhile atrial arrest required 3mM of esmolol. These findings were in line of Bessho's work (Bessho and Chambers 2001). We, then demonstrated that superfusing the atrium has resulted in inducing atrial arrest in some of the hearts at 1 mM. Equally, pacing the ventricle during esmolol arrest did not generate contraction.

CHAPTER 9. SUMMARY OF RESULTS, CONCLUSIONS AND FUTURE WORK

9.1 Esmolol's mechanism of arrest

Since the development of propranolol as a β-blocker in the 1960's, it has been shown to have many indications. These include myocardial protection against ischaemic heart (Mason et al. 1969), treating hypertension (Waal 1966), atrial arrhythmias (Harrison et al. 1965) and hyperthyroidism (Howitt and Rowlands 1966). This resulted in huge development of more selective and non-selective β-blockers. However, the development of the parenteral short acting-β-blocker esmolol has shifted the target patients from chronic therapeutic use to critically ill patients (Zaroslinski et al. 1982), mainly to control heart rate and titrate blood pressure. Shortly after that, its role in mitigating for the injury of myocardial infarction in the acute phase was studied (Lange et al. 1983). Within a few years it became obvious that esmolol had something to offer in the acute setting of myocardial infarction and it was consequently used for the first time by Frazier and Sweeny (Sweeney and Frazier 1992) in emergency surgical revascularisation using minimal ventricular contraction. It was in the acute setting when high doses of esmolol were first used. Since, the main problem with esmolol when given at high doses is hypotension, this was less of a problem during cardiac surgery with minimal ventricular activity because the patient's circulation is already supported by a ventricular assist device (Sweeney and Frazier 1992) or later by conventional cardiopulmonary bypass (Kuhn-Regnier et al. 1999; Scorsin et al. 2003). The negative inotropic and chronotropic effects induced by esmolol had always been assumed to be due to its β-blocking activity. The minimal ventricular activity reported by Sweeney (Sweeney and Frazier 1992) could have been partially explained by its β-blocking effect as blocking the β-receptors in patients could have resulted in a small negative inotropic and chronotropic effect by blocking the systemic effect of the circulating catecholamines. However, in the study by Scorsin and colleagues (Scorsin et al. 2003), esmolol was administered directly into the coronary arteries using a bolus of 250 to 300

mg over a period of 2 to 3 minutes. One can extrapolate that esmolol concentration would have been close to 1 mM, as the coronary flow over this 3 minute- period could not have been more than a litre. This resulted in profound bradycardia (30 bpm) and negative inotropic effect, which was assumed to be due to the β-blocking effect. Again, in this clinical study, esmolol was administered in vivo where there is normally a catecholamine background to, which esmolol could have blocked the cardiac response. However, the use of such high concentrations and the profound bradycardic response does suggest that the mechanism of action of esmolol was beyond blocking the β-receptors in the heart and could possibly be exerting a direct negative inotropic and chronotropic effect. In addition, *in vitro* studies using Langendorff perfused hearts from our lab (Bessho and Chambers 2001; Bessho and Chambers 2002) and others (Ede et al. 1997) in, which complete cardioplegic arrest was achieved with esmolol as an alternative to the conventional cardioplegia could not be explained by blocking the β-receptor in isolation of background catecholamine circulation. Pilot data from our lab using single myocyte confirmed the independence of β-receptors from this effect, and also demonstrated a direct negative inotropic effect of esmolol by inhibiting contraction and Ca_{tr} (Figure 1.14). Importantly, it showed the persistence of smaller amplitude of Ca_{tr} with complete inhibition of contraction. One mechanism that could explain this is desensitisation to calcium. Another possible mechanism was that esmolol blocks the L-type calcium channels as has been suggested by Arlock and colleagues (Arlock et al. 2005) from an observation they made while operating on a patient using off pump beating heart surgery where a bolus of esmolol resulted in profound hypotension and negative inotropic effect necessitating emergency conversion to cardiopulmonary bypass. They studied this effect further by measuring the action potential and observed an inhibition in the plateau phase of the action potential, which is dominated by calcium entry through the $I_{Ca,L}$ channels. Subsequently, they measured the $I_{Ca,L}$ using patch clamping experiments in pig and guinea pig hearts, with results suggesting possible inhibition of $I_{Ca,L}$ (Arlock et al. 2005).

The initial studies (Chapter 3) aimed to reproduce the findings of the pilot data (as in Figure 1.14). We showed that esmolol inhibited contraction in the paced isolated myocytes in a dose-dependent manner, and at 1 mM it inhibited contraction. This inhibitory effect was not altered with background perfusion of atenolol, which suggests

that it is independent from the β-blocking receptors. The calcium transient was also inhibited to a lesser extent than the negative inotropic effect. These observations were in line with the original myocyte pilot data with the exception of being unable to reproduce the persistence in the calcium transient at higher esmolol concentrations (1 mM) when complete arrest was achieved. The mechanism of action was therefore proven to be independent from the β-receptors, and calcium transient inhibition was shown to be instrumental in this negative inotropic effect. Therefore, in subsequent studies in the following chapters, we aimed to explore, by exclusion, the possible mechanism or mechanisms that can induce such effect. In Chapter 4 , we established that the SR was independent from this effect by demonstrating the negative inotropic effect of esmolol in myocytes pre-treated with an SR blocker. The possibility of myofilament desensitisation by esmolol, which was suggested by the pilot data was investigated in Chapter 5; in skinned myocytes esmolol did not alter the response to calcium. This was tested by pre-treating the myocytes with esmolol before skinning, and in a separate experiment when it was added to the activation solution and applied directly to the myofilaments after skinning. In a more physiological preparation of intact myocytes, the relationship between contraction and extracellular calcium was studied in individual myocytes with or without esmolol; again, there was no indication that esmolol had any effect on desensitising the myofilament to calcium. After excluding these two possibilities, we turned to patch clamping techniques and investigated the effect of esmolol on ionic channels in the myocyte membrane. Arlock and colleagues (Arlock et al. 2005) have suggested the possibility of inhibiting the $I_{Ca,L}$ current by measuring the action potential and recorded inhibition in the calcium phase. In Chapter 6 , the effect of esmolol on the L-type calcium channels was studied and this demonstrated that esmolol inhibited the $I_{Ca,L}$ in a dose-dependent manner. The inhibition of the L-type calcium channel would explain the negative inotropic effect, leading to higher doses causing contractile failure and therefore the induction of arrest. However, any further influence of esmolol on other membrane channels cannot be excluded. One particular target that was important to explore is the fast sodium channels; β-blockers were known for their local anaesthetic effect at high doses (Smith 1982). Additionally, in a few experiments on the paced intact myocytes, it was noticeable that the pacing threshold to induce contraction has increased once the

myocytes where superfused with esmolol below the arresting doses, and this can be explained by a local anaesthetic effect (ie. Na-channel blockade). Therefore, the effect of esmolol on the fast sodium channels was examined using the ruptured patch clamping technique (described in Chapter 7); we demonstrated that esmolol inhibited the fast sodium channel current at lower concentrations than those that inhibited the L-type calcium current. We also demonstrated that this effect was not rate or voltage-dependent. Deng and colleagues (Deng et al. 2006) had presented similar results by measuring I_{Na} from hyperpolarising holding potentials of -120 mV compared to the more physiological holding potential of -90mV used in our experiments. Sodium channel blockers are known to have a negative inotropic effect (Honerjager et al. 1986), possibly by reducing the intracellular [Na^+] and subsequently increase the extrusion of calcium from the cell via the sodium/calcium exchanger. This could result in less releasable calcium stored in the SR. It is, therefore, possible that an effect of esmolol on the fast sodium channels contributed to the negative inotropic effect of esmolol by this mechanism. However, we demonstrated that the negative inotropic effect of esmolol was independent from the SR function, which makes it unlikely for the inhibition of fast sodium channels to be the predominant mechanism. As for the arresting effect of esmolol in Langendorff- perfused hearts, we demonstrated that, despite significant inhibition of I_{Na} at 0.3 mM in the isolated myocyte, esmolol failed to arrest the heart at these concentrations. This suggests that the action potential at these concentration is still being generated with very little or no effect from the Na channels. A possible explanation for this could be that the action potential generated using doses between 0.3 and 1 mM concentrations has been through a Na independent mechanism, which was described as the slow action potential where an action potential was generated in paced myocytes (Li and Sperelakis 1983). Even this mechanism does not reliably explain the arrest (or lack of it) using this range of esmolol in the Langendorff rat hearts without considering that the two mechanisms of blocking the sodium and the calcium channels are complimentary to each other. Beyond trying to understand the exact mechanism of esmolol, this also proposed a question of whether the negative inotropic effect observed at submillimolar concentrations without arrest is just a smaller effect of the same phenomenon, which eventually leads to a total arrest at higher concentrations, or whether the arrest and the negative inotropic effect are two separate phenomena. It is

possible that esmolol had induced the negative inotropic effect by targeting the myocyte while the arrest was induced by blocking the pacemaker and the conduction system. The final results chapter aimed at answering this question by defining the arrest and the negative inotropic mechanisms in the Langendorff perfused rat heart. Bessho and colleagues (Bessho and Chambers 2001) demonstrated that esmolol (at 1 mM) induced ventricular arrest with the persistence of atrial activity (Figure 1.13). These findings suggested that esmolol could have induced arrest in these hearts by inhibiting the conduction system only, in particular the AV node. By modifying the Langendorff perfusion model through pacing the ventricle and superfusing the atrium separately, we demonstrated that contraction was inhibited in the ventricle at the level of the myocardium, with arrest not being limited to inhibition of conduction. We also demonstrated that the lack of arrest at the level of the atrium is possibly due to the lack of perfusion.

In conclusion, these studies have demonstrated that esmolol has an inhibitory effect on the fast Na and the L-type calcium channels along with its known effect as a β-blocker in the myocardium. This effect is dependent on the therapeutic concentration as demonstrated schematically in Figure 9.1. The concentration response studies have shown that the inhibition of the L-type calcium channel requires higher concentrations of esmolol than the Na channel blocking effect. These concentrations are closer to the concentrations, which induces arrest to the heart. This strongly suggest that, despite the theoretical importance of I_{Na} arrest in initiating the action potential, blocking the L-type channels was the instrumental mechanism in its negative inotropic effect and most importantly, its cardioplegic arrest.

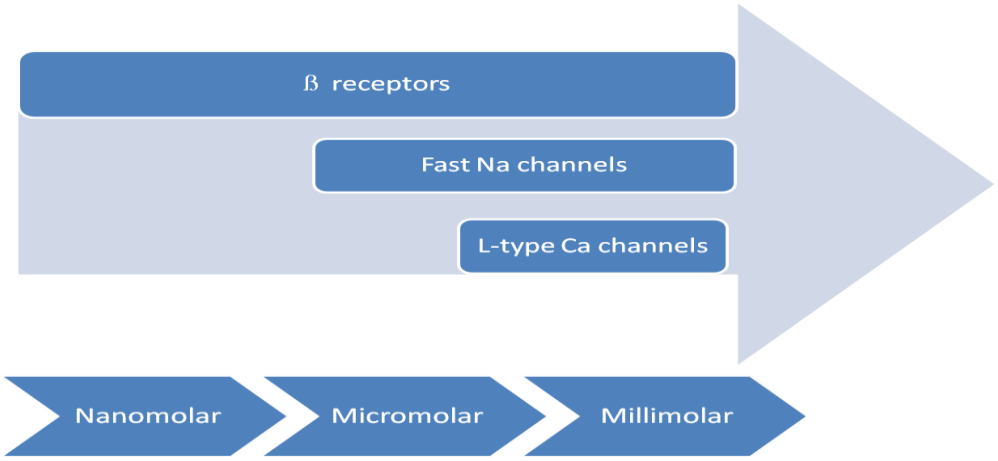

Figure 9.1 Schematic diagram presenting the inhibitory effect of esmolol on the cellular targets in relation to concentration.

9.2 Designing future cardioplegic solutions and the potential application of esmolol in polarising cardioplegia

The introduction of this thesis highlighted the limitations of hyperkalaemia-based cardioplegia (Section1.7.1.1), which has remained the gold standard in ischaemic myocardial protection its development in the since was developed in the 1970's; elevated extracellular potassium depolarises the membrane potential, which inactivates the fast Na^+ channels and thereby blocks conduction of the myocardial action potential. This can lead to myocyte damage from intracellular Na^+ and Ca^{2+} loading, as demonstrated in Figure 1.10. Despite the long standing attempts to find an alternative to hyperkalaemia to overcome its limitations most concepts remained at the experimental and lab stage. We previously indentified certain criteria, which should be met in designing any cardioplegic solution (Section1.6.1 1.6.1) and these can be summarised by the following; the ability to arrest, to have myocardial protective specifications against ischaemia, to be rapidly reversible and to have low toxicity and short half-life. We also identified the possible cellular targets and mechanisms in Section 1.7, which can or have been targeted to induce cardioplegic arrest. These cellular targets and the agents investigated to induce cardioplegic arrest are highlighted in Figure 9.2. In Section 1.7 we reviewed a number of pharmacological agents individually, and

concluded that most of these agents have never been used beyond animal trials. In addition, we concluded that most of these agents would have not met all the criteria identified for a suitable cardioplegic solution. Whilst the majority of these agents were probably able to induce arrest and offer some myocardial protection, there were some concerns about their safety when used in high arresting doses, due to the possibility of slow reversibility and possible accumulation in the blood stream with long lasting effect beyond the cessation of cardiopulmonary bypass (Fallouh et al. 2009). Table 9.1 highlights these criteria and indicates the suitability of the various pharmacological agents investigated or identified as possible cardioplegic agents.

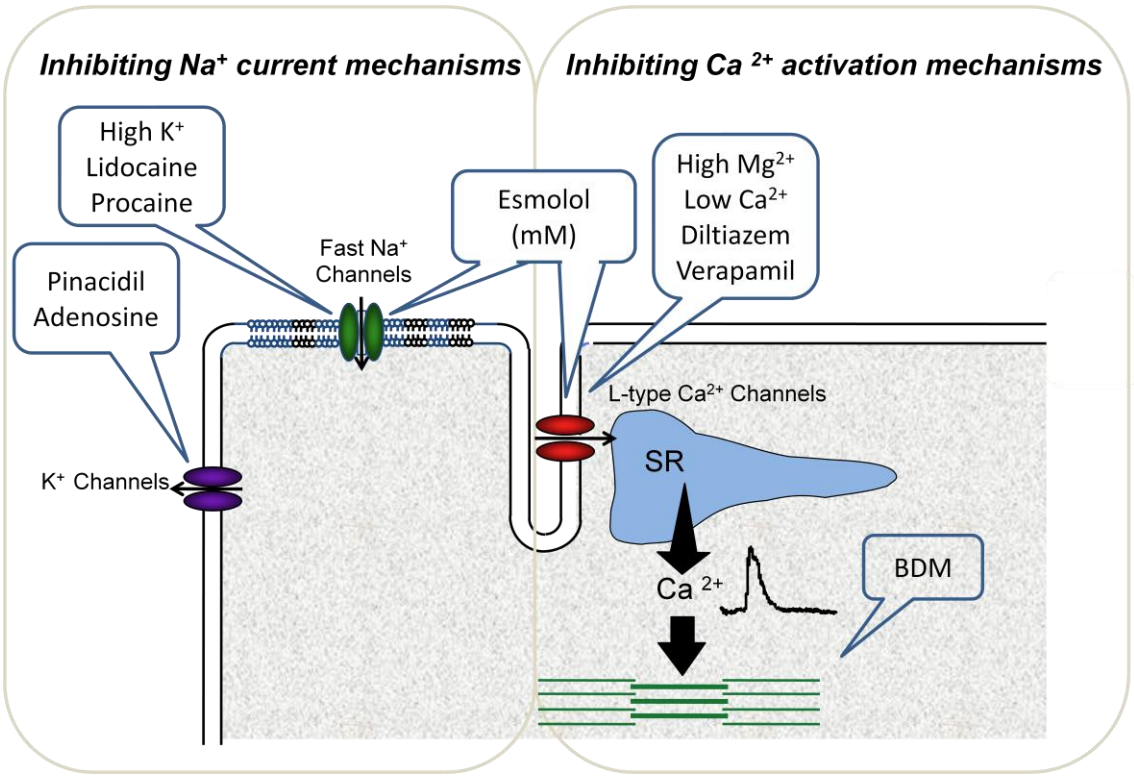

Figure 9.2 Schematic demonstrating the cellular targets and different cardioplegic agents have been studied clinically and experimentally (modified from Figure 1.3 Schematic graph explaining the concept of myocardial protection associated with cardioplegia in prolonging the period of reversible ischaemia. Figure 1.7)

	Lidocaine	*Pinacidil*	*Adenosine*	*Diltiazem/ Verapamil*	*BDM*	*Esmolol*
Diastolic arrest	✓	?	?	✓	?	✓
Myocardial Protection	✓	✓	✓	✓	✓	✓
Reversibility	✓	✓	✓	X	✓	✓
Safety	X	XX	✓	X	?	✓

Table 9.1 The efficacy vs. safety profile of potential pharmacological arrest agents for cardioplegia (✓: suitable, X: not suitable, ?: further evidence is required)

These safety criteria could possibly be a reason why most of these agents were not used clinically as an alternative to conventional cardioplegia. As for esmolol, it was shown to be able to induce cardiac arrest in diastole (Bessho and Chambers 2001), which makes it conceptually suitable to be used as a cardioplegic agent. It was also found to be protective against ischaemia/reperfusion injury (Roth and Torok 1991; Roth et al. 1995), and to be safe and effective as an intermittent cardioplegic agent (at least in *in-vitro* isolated rat hearts) (Bessho and Chambers 2001; Bessho and Chambers 2002). As esmolol has a short half-life due to breakdown by red cell esterases (Erhardt et al. 1983), its clearance does not depend on the liver or the kidneys, which might be compromised during cardiopulmonary bypass. This makes the effect of esmolol significantly reduced before the cessation of cardiopulmonary bypass. It is, therefore, not surprising that esmolol was used as an alternative to cardioplegia in clinical trials with success and in *in-vivo* canine trials mentioned above where continuous coronary perfusion of esmolol was maintained creating profound bradycardia (minimal myocardial contraction) with systemic circulation being supported with cardiopulmonary bypass. Despite the fact that conventional complete cardiac arrest was not achieved with esmolol in these clinical studies, this application was proven to be a

safe and effective alternative to conventional blood cardioplegia (Kuhn-Regnier et al. 1999; Geissler et al. 2000; Scorsin et al. 2003). It is possible that complete arrest using high doses of esmolol might not confer the same protection it offered when it was used for minimal ventricular activity, as we have shown (Section 8.3.4) that the best reversibility for LDVP after washing out esmolol in the Langendorff hearts was 0.3 mM (Figure 8.6); a concentration lower than the arresting effect. This poses the question regarding the possibility of achieving arrest by combining another agent, which can offer synergistic arresting effect to esmolol at doses below the arresting concentrations in order to benefit from the rapid reversibility of lower doses of these agents. The agents that are likely to offer such effect are expected to target different cellular targets from esmolol and at the same time should be meeting the criteria set above. Therefore, this excludes all other sodium and calcium channel blockers and narrows the choice to the remaining two mechanisms; potassium channel activation and myofilament desensitisation (Figure 9.2). By referring to Table 9.1, one can conclude that adenosine looks to be the most suitable agent to combine with esmolol in order to enhance the arresting effect of esmolol and possibly other myocardial protective effects. We can also argue that the addition of a myofilament desensitiser like BDM could enhance the arresting and myocardial protective effect further. However, BDM long-term safety and its toxicological burden on patients after weaning the cardiopulmonary bypass has to be established as the information on this is very limited in this context. As a conclusion, a cardioplegic preparation can therefore be designed with targeting these multiple cellular mechanisms without ignoring the criteria highlighted above.

Interestingly, a cardioplegic preparation, which contains esmolol and adenosine is likely to induce an arrest by blocking both sodium and calcium channels and also activating the $K_{(Ach)}$ channels in the SA node without depolarising the myocyte membrane. This is, therefore, likely to be a polarising cardioplegic preparation, which can be a viable alternative to the depolarising St. Thomas' Hospital cardioplegia.

The following chapter will explore this principle, and will provide preliminary data for future studies in designing a clinically viable polarising cardioplegic solution, which can overcome the limitations of the current St. Thomas' Hospital Cardioplegia.

9.3 **Limitations of experimental models**

This PhD examines the effect of arrest by esmolol in the rat hearts in physiological rather than pathological settings which is usually different in patients coming in cardiac surgery. Additionally, patients undergoing cardiac surgery are usually already on a variety of cardiac active agents which can be β-blockers, calcium channel blockers potassium openers or even Na channel blockers. This could interact with any possible effect of esmolol in these hearts. Finally, and as explained earlier in Chapter 1 the rat heart blood supply is different from the human by the dual intra and extra-cardiac blood supply (Halpern 1957), meanwhile in the Langendorff perfused model the blood supply is mainly through the coronary arteries and we assume that there is no regional ischaemia is sustained because of that and there is adequate collateral supply between the two networks.

In terms studying the effect of esmolol on the isolated myocytes, these experiments are performed in non-physiological conditions and settings; for instance the calcium sensitive Fura 2 which was used in Chapters 3 and 4 is known to have a chelating effect to calcium which could potentially have an added negative inotropic effect. Meanwhile, the patch clamping studies were designed to demonstrate the current in an artificial setting like hyperpolarising the cell before activating the Na current, equally many non-physiological compounds to dilute the cytosol. However, all these methods are well established investigation tools which should provide reliable information.

CHAPTER 10. RESULTS 7: ESMOLOL ADENOSINE CARDIOPLEGIA (PILOT DATA)

10.1 Introduction

We have previously demonstrated that 1 mM esmolol can offer cardioplegic arrest through blocking the L-type calcium channels and the fast Na-channels. This is likely to be polarising arrest as the extracellular K^+ is not being altered. We also suggested that esmolol can be a clinically suitable cardioplegic solution (Fallouh and Chambers 2007; Fallouh et al. 2009) due to its short half life and its breakdown in the red cells. In Section 8.3.4 we demonstrated that the reversibility of esmolol at 0.3 mM was superior to the 1 mM concentration presented by quicker and higher LVDP recovery and heart rate (Figure 8.6 Figure 8.7). Esmolol at concentrations 0.3 mM, however, did not arrest the heart reliably. The possibility of combining esmolol with another agent could be a successful strategy to overcome this limitation and offer suitable to be used clinically as a cardioplegic solution. After systematic review exploring all agents explored in the previous chapter (Section9.2), adenosine was identified to be a suitable pharmacological agent to enhance the arresting and the myocardial protection effects of esmolol at lower concentrations, which could offer faster reversibility. We intended in this final chapter to evaluate the concept of combining esmolol and adenosine in a polarising cardioplegic solution, which aimed to induce arrest by influencing multiple cellular targets. This study was performed under my direct supervision by a medical student during the summer holiday and intended to assess the combination as a proof of concept in designing the future polarising cardioplegia. Similar solution was designed by Dobson and colleagues (Dobson and Jones 2004) using a combination of lidocaine and adenosine and was shown to be a very effective arresting solution with protection superior the current gold standard solution, St. Thomas' Hospital cardioplegia. However esmolol and Lidocaine has one limitation; this solution can be administered in limited quantities due to the possibility of lidocaine systemic accumulation and toxicity (Corvera et al. 2005; Fallouh and Chambers 2007). This limitation is not shared by

esmolol and adenosine combination as explained earlier (Section9.2). This experiment assesses the effectiveness of the arrest and the protective specification of an esmolol and adenosine combination compared with the lidocaine and adenosine combination and with the conventional St. Thomas' Hospital Cardioplegia.

10.2 **Methodology**

10.2.1 Langendorff perfusion

The isolation of the rat hearts and the Langendorff perfusion methodology is explained in details in Sections 2.2.7 and 2.5. In brief, after rapidly excising the heart it was attached to the Langendorff perfusion system and perfused with KHB solution perfused within 30 to 60 seconds to reduce the length of ischaemia. After removing the left atrium, the ventricular balloon was inserted through into the left ventricle. The heart is maintained in a water-jacketed glass reservoir covered with paraffin film 'parafilm' to maintain the temperature with a thermometer probe in the heart chamber to ensure the desired temperature is maintained. The heart was then perfused with KHB perfusion at 37^{o}C at pressure of 100 cm of water. An intra ventricular balloon was adjusted by filling the balloon to achieve an end-diastolic pressure of around 4-6 mmHg. A surface ECG lead was attached to the surface of the ventricle to record a unipolar recording and was left to stabilise for 20 minutes. The hearts were then randomised to the desired treatment and the desired temperature and the perfusion pressure for all treatments was always maintained at 100 cm of water. Values were, again, presented as mean ± standard error of mean (SEM) after normalising to control, which was measured after the stabilisation period unless stated otherwise. Esmolol, adenosine and lidocaine were prepared by adding the desired concentration to the KHB solutions.

10.2.2 Dose combination and ischaemic protocol

In this study, our aim to identify the combination doses that deliver a reliable and timely arrest with a target esmolol concentration below 1 mM and as close to 0.3 mM as possible, based on the washout data presented in Section 8.3.4. Meanwhile, studies from Dobson and colleagues (Dobson and Jones 2004) have demonstrated that an adenosine dose range around 0.2 mM would offer maximum protection and effective arrest as an

additive to the fast sodium channel blocker, lidocaine. Preliminary studies on a single heart concluded that esmolol at 0.3 mM and adenosine at 1 mM failed to arrest the heart; it became clear that increasing the concentration of esmolol would be required in order to achieve an effective arrest. We opted to use 0.6 mM of esmolol and run a dose response study to assess the time to achieve arrest when combined with the adenosine at a range of the following doses; 0.125, 0.25, 0.5 and 1 mM. Studies were conducted using 4 hearts; each heart was randomised to the 4 groups of adenosine concentrations combined with esmolol (0.6 mM). The times to full arrest were 70, 53, 50 and 38 seconds, respectively. We chose the combination of esmolol 0.6 mM and adenosine 0.25 mM as the optimal concentration to conduct the following preliminary studies to assess the suitability and effectiveness of the combination as a cardioplegic solution.

St. Thomas' Hospital Cardioplegia (STH2) is remained the gold standard whereas lidocaine (0.6mM) and adenosine (0.2mM) cardioplegia (LAC) was shown to offer superior protection over STH2 (Dobson and Jones 2004). We aimed to design an ischaemic protocol, which has the best chance to demonstrate any possible superiority of esmolol and adenosine combination as cardioplegia (EAC) over STH2 and LAC, and can translate to the clinical environment. Therefore, we opted to mimic the situation during complex operations where long periods of ischaemia can be unavoidable. This has become the trend in the way the profile of cardiac surgery recently as patients are becoming older and sicker with multiple pathology (Parissis 2011) and, hence, better myocardial protection is more important than ever in these situations. In order to address this need, we used a prolonged period of ischaemia (four hours) with multiple cardioplegic infusion (3 minutes) at intervals of 30 minutes instead of the classical 20 minute-interval. This is, again, one of the situations that surgeons are facing more frequently when dealing with multiple valves or major aortic surgery, when occasionally, it is not technically feasible to be able to give cardioplegia religiously ever 20 minutes as this could lead in interruption of the surgical flow and results in overall further delay in the ischaemic time. Equally, the general practice in such procedures is to use topical cooling on the heart during ischaemia and to use cold cardioplegia (Robinson et al. 1995). Therefore, we used room temperature cardioplegia in a crystalloid preparation and the heart was maintained at room temperature during ischaemia. After the stabilisation period 18 hearts were randomised into treatment with

either STH2, EAC or LAC. 6 hearts in each group (n=6). After 4 hours ischaemia with multiple cardioplegic administration at 30 minutes interval the hearts were perfused with KHB solution. Time to arrest, EDP during ischaemia (ischaemic contracture), heart rate recovery and LVDP recovery were recorded. Statistical analysis is performed by using 2 way ANOVA and Bonferroni post-hoc test.

10.3 **Results**

10.3.1 Esmolol adenosine cardioplegia vs. lidocaine adenosine cardioplegia and STH2

The time to arrest was found to be significantly different between the three groups; 52±14, 13±3* and 8±1.9* seconds for EAC, STH2 and LAC respectively (* P<0.001). Ischaemic contracture was measured by recording the maximum intra-ventricular pressure recorded during the ischaemic period. The maximum EDP values were The highest contraction was seen in the STH2 group and these values were 9.8±1.4, 18.3±4.5 and 76.3±16.8* mmHg for EAC, LAC and STH2 respectively found to be also significantly different between the three groups P<0.01. These data are highlighted in Figure 10.1.

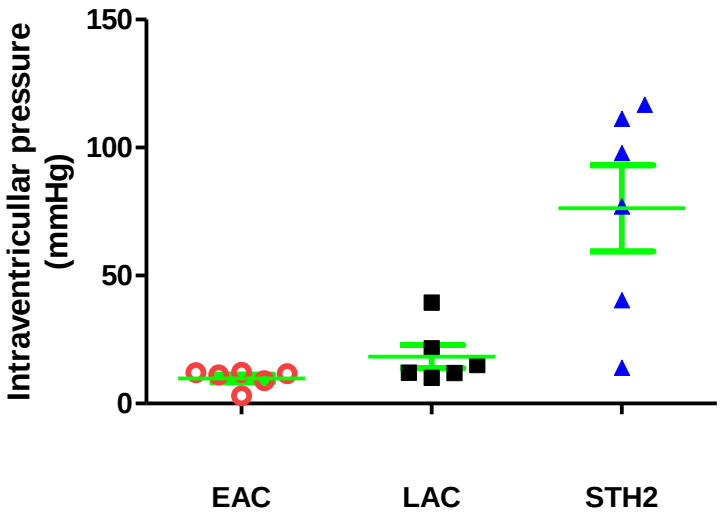

Figure 10.1 Maximum intra-ventricular pressure measured during ischaemic arrest as an indication for ischaemic contraction (n=6)

Recovery of heart rate and LVDP were measured during the reperfusion period at 5 to 10 minutes intervals and the values were normalised to the control period. The heart rate recovery data are shown in Figure 10.2; slightly slower recovery in heart rate was observed in both EAC and STH2 compared to the LAC group, but after 30 minutes of perfusion the three groups become similar. Recovery of LVDP showed significant improvement during reperfusion in the EAC group compared with the STH2 group (Figure 10.3).

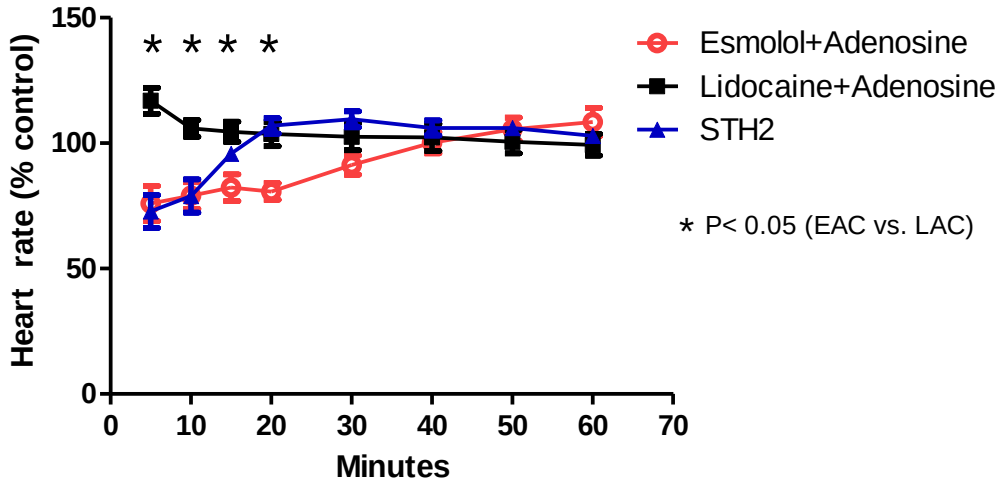

Figure 10.2 Heart rate recovery during reperfusion between the three groups demonstrating the difference in heart rate recovery at early perfusion between the EAC group and the LAC group.

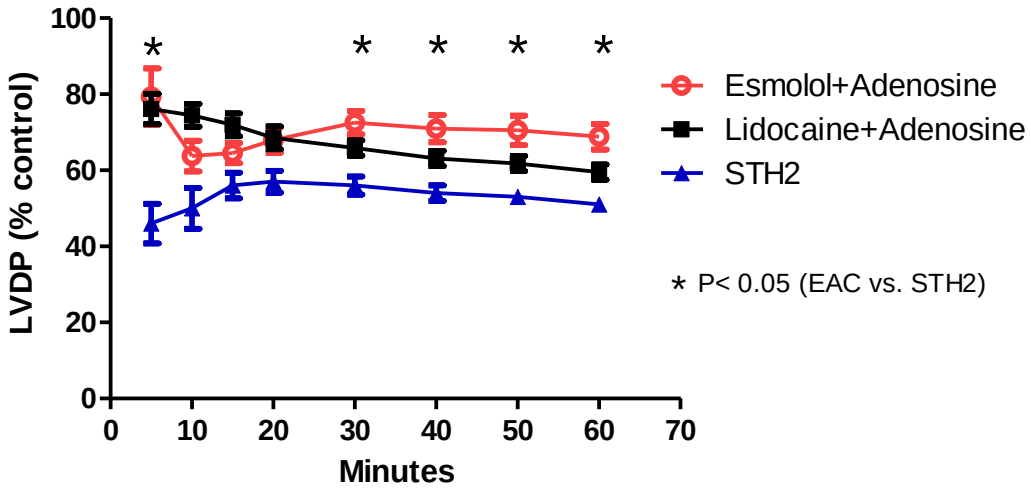

Figure 10.3 LVDP recovery during reperfusion between the three group demonstrating the difference in contraction recovery at early perfusion and later between EAC and STH2 group.

10.4 **Discussion and conclusion**

After establishing the exact mode of action of esmolol, we aimed to follow a systematic method of design for a cardioplegic solution that would fit the criteria we previously established to determine the suitability of any cardioplegia. We aimed to use agents with known protective effects against ischaemia and, by targeting multiple cellular targets to induce arrest, we were able to use smaller doses of each pharmacological agent (Fallouh et al. 2009). This systematic approach yielded the combination of esmolol and adenosine. Blocking the sodium and the calcium channels by esmolol along with hyperpolarising the AV node by adenosine should induce arrest without depolarisation. We can, therefore, consider this combination as a non-depolarising cardioplegic solution. Our aim in these studies was to examine the concept of whether we can develop a cardioplegic solution, which can offer better protection against ischaemia. Due to time limitations, further studies of other potential agents that might enhance other myocardial protection (such as magnesium or BDM) were not investigated. It does appear, however, that the combination does offer a viable option as a polarising cardioplegic solution in the Langendorff perfused rat heart compared with the current

gold standard cardioplegic solution. It also was comparable to the effect of a lidocaine and adenosine combination; this solution was shown to be superior to STH (Dobson and Jones 2004; Corvera et al. 2005) with the potential possibility of lidocaine accumulation if used liberally in at high concentrations (Corvera et al. 2005; Fallouh and Chambers 2007). Esmolol and adenosine combination can overcome this issue by offering a safe and effective polarising cardioplegic solution with superior myocardial protection. Further work will be conducted to expand the potential of this adenosine-esmolol combination in offering improved myocardial protection.

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